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(54) Title: METHODS FOR USING GRANZYMES AND BINDING MOLECULES THEREOF FOR TREATING DISEASES CHARACTERIZED BY ABNORMAL APOPTOSIS

(57) Abstract

A method for determining if an animal is at risk for a disease resulting in abnormal apoptosis is described. An animal is provided and an aspect of metabolism or structure of a serine protease, e.g., a granzyme, or a serine protease binding molecule in the animal is evaluated. An abnormality in the aspect of the metabolism or structure is diagnostic of being at risk for a disease resulting in abnormal apoptosis. Also described are methods for evaluating an agent for use in modulating apoptosis, methods for effecting or inhibiting apoptosis in a cell, and methods for treating unwanted cell or infectious particle proliferation or treating an autoimmune disease or a transplant graft rejection in an animal. Pharmaceutical compositions are also provided.

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METHODS FOR USING GRANZYMES AND BINDING MOLECULES THEREOF FOR TREATING DISEASES CHARACTERIZED BY ABNORMAL APOPTOSIS

This application claims the benefit of U.S. Provisional Application No. 60/056,333 filed August 18, 1997.

The U.S. Government has a paid-up license in this invention and the right in limited circumstances to require the patent owner to license others on reasonable terms as provided for by the terms of Grant No. K08 CA01449 awarded by the National Institutes of Health/National Cancer Institute.

Field of the Invention

This invention relates generally to treatments, diagnoses and therapeutic agents for diseases resulting in abnormal apoptosis.

Background of the Invention

The process of programmed cell death or apoptosis is a genetically determined cellular program. It consists of a stereotypic sequence of events characterized by nuclear condensation, cellular membrane blebbing, and DNA degradation. Apoptosis plays a vital role in normal development and tissue remodeling, escape from cellular transformation, and the immune response. Apoptosis can be initiated by a variety of exogenous factors, e.g., radiation, oxidative stress, or chemotherapeutic agents, or by natural endogenous processes, e.g., cytotoxic T lymphocytes, corticosteroids, or engagement of cell surface molecules such as fas or TNF. Apoptosis is particularly important for protection against intracellular pathogens and in tumor immunosurveillance. Unwanted apoptosis is associated with certain autoimmune diseases and transplant graft rejection.

There is a need for therapeutic treatments which activate apoptosis so as to inhibit diseases associated with unwanted cell or infectious particle proliferation, as well as a need for therapeutic treatments which inhibit apoptosis so as to treat certain autoimmune diseases and transplant graft rejections.

Summary of the Invention

It is an object of the invention to utilize serine proteases, e.g., granzymes, and serine protease binding molecules to aid in the treatment, diagnosis and/or identification of therapeutic agents for diseases characterized by abnormal apoptosis.

It is an object of the invention to modulate apoptosis by utilizing an agent which affects

the metabolism or structure of granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27.

In one aspect, the invention features a method for determining if an animal is at risk for a disease resulting in abnormal apoptosis. An animal is provided. An aspect of the metabolism or structure of a serine protease, e.g., a granzyme, e.g., granzyme A, or a serine protease binding molecule, e.g., a granzyme binding molecule, e.g., a granzyme A binding molecule, e.g., PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27, is evaluated in the animal. An abnormality in the aspect of metabolism or structure is diagnostic of being at risk for a disease resulting in abnormal apoptosis.

Another aspect of the invention is a method for evaluating an agent for use in modulating apoptosis. A cell is provided. An agent is provided. The agent is administered to the cell in a therapeutically effective amount. The effect of the agent on an aspect of the metabolism or structure of a serine protease or a serine protease binding molecule is evaluated. A change in the aspect of the metabolism or structure is indicative of the usefulness of the agent in modulating apoptosis.

Another aspect of the invention is the agent so identified.

Another aspect of the invention is a method for effecting apoptosis in a cell. A cell which is deficient in effecting apoptosis is provided. An effective amount of an active endogenous DNase capable of degrading genomic DNA in the cell is provided. The DNase is administered to the cell so as to effect apoptosis.

Another aspect of the invention is a method for inhibiting apoptosis in a cell. A cell which is capable of effecting apoptosis is provided. An agent which inhibits genomic DNA degradation in the cell by an endogenous DNase is provided. The agent is administered to the cell so as to inhibit apoptosis in the cell.

Another aspect of the invention is a method for treating unwanted cell or infectious particle proliferation in an animal. An animal having cells in need of treatment for unwanted cell or infectious particle proliferation is provided. An agent capable of activating apoptosis is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the unwanted cell or infectious particle proliferation in the animal occurs.

Another aspect of the invention is a method for providing an animal having cancer with a therapeutic level of a polypeptide, e.g., granzyme A, a binding molecule for granzyme A, e.g., PHAP I, PHAP II, a complex comprising PHAP II, heat shock protein 27, or a biologically active analog or fragment thereof. The polypeptide is provided to the animal by administering to the

animal a nucleic acid encoding the polypeptide.

Another aspect of the invention is a method for treating an animal at risk for unwanted cell or infectious particle proliferation. An animal at risk for unwanted cell or infectious particle proliferation is provided. An agent capable of activating apoptosis is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the unwanted cell or infectious particle proliferation occurs.

Another aspect of the invention is a pharmaceutical composition for treating unwanted cell or infectious particle proliferation in an animal comprising a therapeutically effective amount of an agent capable of effecting apoptosis, e.g., a serine protease, a binding molecule for a serine protease, or a biologically active analog or fragment thereof, and a pharmaceutically acceptable carrier.

Another aspect of the invention is a pharmaceutical composition for treating unwanted cell or infectious particle proliferation in an animal comprising a therapeutically effective amount of a recombinant nucleic acid encoding a polypeptide capable of effecting apoptosis, e.g., a serine protease, a binding molecule for a serine protease, or a biologically active analog or fragment thereof, and a pharmaceutically acceptable carrier.

Another aspect of the invention is a method for treating an autoimmune disease or a transplant graft rejection in an animal. An animal in need of treatment for an autoimmune disease or a transplant graft rejection is provided. An agent which inhibits apoptosis is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the autoimmune disease or transplant graft rejection occurs.

Another aspect of the invention is a method for treating an autoimmune disease or a transplant graft rejection in an animal. A therapeutically effective amount of an agent capable of inhibiting DNase activity of a complex comprising PHAP II or a component of the complex, or capable of inhibiting granzyme A cleavage of PHAP II, is administered to an animal having an autoimmune disease or a transplant graft rejection.

Another aspect of the invention is a method for treating an animal at risk for an autoimmune disease or a transplant graft rejection. An animal at risk for an autoimmune disease or a transplant graft rejection is provided. An agent capable of inhibiting apoptosis is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the autoimmune disease or transplant graft rejection occurs.

Another aspect of the invention is a pharmaceutical composition for treating an autoimmune disease or a tissue graft rejection in an animal comprising a therapeutically effective

amount of an agent capable of inhibiting apoptosis by inhibiting a serine protease, a binding molecule for a serine protease, or a biologically active analog or fragment thereof, and a pharmaceutically acceptable carrier.

Another aspect of the invention is a pharmaceutical composition for treating an autoimmune disease or a transplant graft rejection in an animal comprising a therapeutically effective amount of a recombinant nucleic acid encoding a polypeptide capable of inhibiting apoptosis by inhibiting a serine protease, a binding molecule for a serine protease, or a biologically active analog or fragment thereof, and a pharmaceutically acceptable carrier.

Another aspect of the invention is a pharmaceutical composition for treating an autoimmune disease or a transplant graft rejection in an animal comprising a therapeutically effective amount of cells wherein a recombinant nucleic acid encoding a polypeptide capable of inhibiting apoptosis by inhibiting a serine protease, a binding molecule of a serine protease or a biologically active analog or fragment thereof, has been introduced into the cells so as to express the polypeptide, and a pharmaceutically acceptable carrier.

Another aspect of the invention is a method of degrading nucleic acid in vitro. Nucleic acid is provided. An isolated complex comprising PHAP II is provided. The nucleic acid is contacted with the complex in vitro such that the nucleic acid is degraded by the complex comprising PHAP II.

Another aspect of the invention is a method of degrading nucleic acid in vivo. A cell having nucleic acid that is deficient in active endogenous DNase for apoptosis is provided. A complex comprising PHAP II is provided. The nucleic acid is contacted with the complex in vivo such that the nucleic acid is degraded by the complex.

Another aspect of the invention is a method for identifying an enzyme substrate. An enzyme having an inactive active site is provided. A composition having a candidate substrate for the enzyme is provided. The composition is contacted wtih the enzyme and it is determined, e.g., by affinity chromatography, if the candidate substrate in the composition binds to the enzyme so as to identify the candidate substrate as a substrate for the enzyme.

Another aspect of the invention is a bacterial recombinant expression vector comprising a nucleic acid encoding granzyme A, a leader sequence for periplasmic export of the granzyme A, and a carboxy terminal tag for purification of the granzyme A. In certain embodiments, the vector also has an enterokinase site 5' of the nucleic acid encoding the granzyme A so as to enable in vitro activation of the granzyme A.

Another aspect of the invention is a cell having a recombinant expression vector

described above.

Yet another aspect of the invention is a method for producing granzyme A comprising culturing a cell having a recombinant expression vector described above under conditions that permit expression of the granzyme A.

The above and other features, objects and advantages of the present invention will be better understood by a reading of the following specification in conjunction with the drawings.

Brief Description of the Drawings

Fig. 1 depicts a recombinant plasmid for Pro-rGranA and a recombinant plasmid for S->rGranA.

Detailed Description

This invention provides a method for determining if an animal is at risk for a disease resulting in abnormal apoptosis. An animal is provided. An aspect of the metabolism or structure of a serine protease or a serine protease binding molecule is evaluated in the animal. An abnormality in the aspect of metabolism or structure is diagnostic of being at risk for a disease resulting in abnormal apoptosis.

By animal is meant human as well as non-human animals. Non-human animals include, e.g., mammals, birds, reptiles, amphibians, fish and insects. Preferably, the non-human animal is a mammal, e.g., a rabbit, a rodent, e.g., a mouse, rat or guinea pig, a primate, e.g., a monkey, or a pig. An animal also includes transgenic non-human animals. The term transgenic animal is meant to include an animal that has gained new genetic information from the introduction of foreign DNA, i.e., partly or entirely heterologous DNA, into the DNA of its cells; or introduction of a lesion, e.g., an in vitro induced mutation, e.g., a deletion or other chromosomal rearrangement into the DNA of its cells; or introduction of homologous DNA into the DNA of its cells in such a way as to alter the genome of the cell into which the DNA is inserted, e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout or replacement of the homologous host gene or results in altered and/or regulatable expression and/or metabolism of the gene. The animal may include a transgene in all of its cells including germ line cells, or in only one or some of its cells. Transgenic animals of the invention can serve as a model for studying apoptosis or for evaluating agents to treat diseases resulting in abnormal apoptosis. In certain embodiments, the determination for being at risk for a disease resulting in abnormal apoptosis is done in a prenatal animal.

By apoptosis is meant programmed cell death. The stereotypic response of apoptosis involves characteristic nuclear condensation, cellular membrane blebbing and DNA degradation. The release of cytotoxic granule contents by cytotoxic T lymphocytes (CTL) can trigger apoptotic target cell death. CTL granules contain a pore forming protein, perforin, and a group of serine proteases termed granzymes, in a proteoglycan matrix. After antigen-specific recognition by the CTL T cell receptor, the cytolytic granules migrate to the region of CTL apposition to its target cell, fuse to the CTL plasma membrane, and release their contents into the intercellular space between the CTL and its target. Perforin is thought to poke holes in the target cell allowing the granzymes to enter and initiate apoptotic cell death via activation of endogenous endonucleases.

Seven mouse, three rat and five human granzymes have been described. (Jenne and Tschopp, Immunol. Revs. 103:53-71 (1988). It has been reported that granzyme A, granzyme B and perforin are the major effectors of granule mediated lysis, and that granzyme A and B act independently and synergistically to induce target cell lysis. (Nakajima et al., J. Exp. Med. 181:1037-1046 (1995). Granzyme A is the most abundant of the proteases in human CTL granules. Granzyme A is a tryptic protease which cleaves synthetic substrates with Lys or Arg at the P1 position. Like granzyme B, it is produced in CTL as a proenzyme, which can be activated by cathepsin C cleavage of a dipeptide. Granzyme B cleaves members of the ICE/ced-3 family of cysteine proteases, thereby activating a ubiquitous apoptotic cascade.

Abnormal apoptosis can result in, e.g., the activation of cell death or the inhibition of cell death. The risk for a disease resulting in abnormal apoptosis that is determined can be a reduced risk or an increased risk as compared to a normal animal.

A serine protease of this invention is meant to include, e.g., the granzymes, proteins involved in coagulation and fibrinolysis, complement proteins, and proteases in other blood cells, e.g., neutroprils and mast cells. Preferred serine proteases are granzymes. A preferred granzyme is granzyme A.

By a serine protease binding molecule is meant any molecule which binds to a serine protease, e.g., a substrate, a regulatory element, a structural component, a chaperone (transport) molecule, or any other type of ligand. A serine protease binding molecule can be, e.g., a polypeptide or a nucleic acid, e.g., RNA or DNA. Preferred serine protease binding molecules are granzyme binding molecules. A preferred granzyme binding molecule is a granzyme A binding molecule. Examples of serine protease binding molecules that have been identified by this invention include PHAP I, PHAP II and heat shock protein 27.

A serine protease binding molecule is also meant to include a complex comprising a serine protease binding molecule. The serine protease binding molecule can be complexed to one or more other molecules, e.g., additional binding molecules, by non-covalent or covalent means. These other molecules include, e.g., polypeptides or nucleic acids, e.g., RNA or DNA.

PHAP I and PHAP II are granzyme A-binding cytoplasmic proteins having molecular weights of 33 and 44 kD, respectively. PHAP I and PHAP II were previously identified as putative HLA-associated proteins. These PHAP proteins are ubiquitously expressed. PHAP I binds to granzyme A, though it is not a substrate for granzyme A. See Example 6. PHAP II is a substrate for granzyme A. See Examples 8 and 9. Granzyme A cleaves PHAP II (44 kD) into a 25 kD product. PHAP II or its cleavage protein can be present, e.g., as a complex comprising a second 25 kD protein and optionally RNA. This complex has active endogenous DNase activity, which can degrade double stranded DNA in a sequence independent manner. See Example 9. In certain embodiments, a component of the complex comprising PHAP II has endogenous DNase activity. As a result of CTL attack, PHAP II or a complex comprising PHAP II migrates from the cytoplasm into the nucleus of the cell where PHAP II is cleaved. See Example 8. The term "a complex comprising PHAP II" as used herein means the complex as described in Example 9.

Heat shock protein 27 and its dimer p53 bind to granzyme A, though they are not substrates for granzyme A. See Example 10. Heat shock protein 27 may be involved in the morphological changes observed with granzyme A loading of target cells and/or may act as a chaperone (transport) molecule for granzyme A and/or the PHAPS or PHAP complexes to the nucleus during CTL attack.

In preferred embodiments, the serine protease is a granzyme, preferably granzyme A, and the serine protease binding molecule is a granzyme binding molecule, preferably a granzyme A binding molecule, and more preferably is PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27.

The polypeptides of this invention, e.g., the serine proteases and certain of the serine protease binding molecules, are meant to include, e.g., natural polypeptides, recombinant polypeptides and synthetic polypeptides. The terms polypeptides, proteins and peptides are used interchangeably herein.

By metabolism of a serine protease or a serine protease binding molecule is meant any aspect of the production, release, expression, function, action, interaction or regulation of these polypeptides. The metabolism of these polypeptides includes modifications, e.g., covalent or non-covalent modifications, of these polypeptides. The metabolism of these polypeptides

includes modifications, e.g., covalent or non-covalent modifications, that these polypeptides induce in other substances. The metabolism of these polypeptides also includes changes in the distribution of these polypeptides, and changes these polypeptides induce in the distribution of other substances.

Any aspect of the metabolism of a serine protease or a serine protease binding molecule can be evaluated. The methods used are standard techniques known to those skilled in the art and can be found in standard references, e.g., Ausubel et al., ed., Current Protocols in Mol. Biology, New York: John Wiley & Sons, 1990; Kriegler, M., ed., Gene Transfer and Expression, Stockton Press, New York, NY, 1989. Preferred examples of the metabolism that can be evaluated include the binding activity of these polypeptides to a binding molecule; the transactivation activity of these polypeptides on a target gene; the DNase or RNase activity of these polypeptides or of complexes containing any of these polypeptides; the chaperone (transport) activity of these polypeptides; the level of these polypeptides; the level of mRNA for these polypeptides; and the level of modifications of these polypeptides, e.g., phosphorylation, glycosylation or acylation.

By binding molecule is meant any molecule to which either the serine protease or serine protease binding molecule can bind, e.g., a nucleic acid, e.g., a DNA regulatory region, a polypeptide, a metabolite, a peptide mimetic, a non-peptide mimetic, an antibody, or any other type of ligand. A binding molecule can be, e.g., a substrate, a regulatory element, a structural component, or a chaperone (transport) molecule. Binding of a serine protease or a serine protease binding molecule to a second binding molecule can be shown by standard methods known to those skilled in the art, e.g., by antibodies against the serine protease, the serine protease binding molecule, or the second binding molecule, or by affinity chromatography, affinity coelectrophoresis (ACE) assays, or ELISA assays.

Transactivation of a target gene by either the serine protease or serine protease binding molecule can be determined, e.g., in a transient transfection assay in which the promoter of the target gene is linked to a reporter gene, e.g., β-galactosidase or luciferase, and co-transfected with an expression vector having a serine protease or serine protease binding molecule gene. Such evaluations can be done in vitro or in vivo.

DNase activity of the serine protease, the serine protease binding molecule, or a complex containing serine protease binding molecule, can be measured by in vitro degradation of soluble DNA visualized by radiolabeling or EtBr staining after agarose electrophoresis, PFGE of genomic DNA, or SDS-DNA-PAGE analysis where calf thymus DNA is incorporated into SDS-

PAGE gels, proteins are renatured in the presence of Ca^{+2} and Mg^{+2} and degraded DNA is visualized by absence of EtBr staining. See Example 9.

The chaperone (transport) activity of the serine protease or binding molecules can be measured by immunofluorescence microscopy, immunoelectron microscopy or immunoblot SDS-PAGE analysis of subcellular fractions of cells.

Levels of protein, mRNA or modifications of the serine protease or serine protease binding molecule can be measured, e.g., in a sample, e.g., a tissue sample, by standard methods known to those skilled in the art.

In certain embodiments, an aspect of the structure of the serine protease or serine protease binding molecule is evaluated, e.g., gene structure or protein structure. For example, primary, secondary or tertiary structures can be evaluated. For example, the DNA sequence of the gene is determined and/or the amino acid sequence of the protein is determined. Standard cloning and sequencing methods can be used as are known to those skilled in the art. In certain embodiments, the binding activity of an antisense nucleic acid with the cellular mRNA and/or genomic DNA is determined using standard methods known to those skilled in the art so as to detect the presence or absence of the target mRNA or DNA sequences to which the antisense nucleic acid would normally specifically bind.

The invention also includes a method for evaluating an agent for use in modulating apoptosis. A cell is provided. An agent is provided. The agent is administered to the cell in a therapeutically effective amount. The effect of the agent on an aspect of the metabolism or structure of a serine protease or a serine protease binding molecule is evaluated. A change in the aspect of the metabolism or structure is indicative of the usefulness of the agent in modulating apoptosis.

In certain embodiments, the method employs two phases for evaluating an agent for use in modulating apoptosis, an initial in vitro phase and then an in vivo phase. The agent is administered to the cell in vitro, and if a change in an aspect of the metabolism or structure of the serine protease or serine protease binding molecule occurs, then the agent is further administered to an animal in a therapeutically effective amount and evaluated in vivo for an effect of the agent on an aspect of the metabolism or structure of a serine protease or a serine protease binding molecule.

By cell is meant a cell or a group of cells, a cell culture, or a cell that is part of an animal. The cell can be a human or non-human cell. Cell is also meant to include a transgenic cell. The cell can be obtained, e.g., from a culture or from an animal. Animals are meant to include, e.g.,

natural animals and non-human transgenic animals. In certain embodiments, the transgenic cell or non-human transgenic animal has a serine protease or serine protease binding molecule transgene, or fragment or analog thereof. In certain embodiments, the transgenic cell or non-human transgenic animal has a knockout for the serine protease or serine protease binding molecule gene.

The cell can have a wild type pattern or a non-wild type pattern for the metabolism or structure of the serine protease or serine protease binding molecule. Preferably, the serine protease is a granzyme, preferably granzyme A, and the serine protease binding molecule is a granzyme binding molecule, preferably a granzyme A binding molecule, and more preferably is PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27. A non-wild type pattern can result, e.g., from under-expression, over-expression, no expression, or a temporal, site or distribution change. Such a non-wild type pattern can result, e.g., from one or more mutations in the serine protease or serine protease binding molecule gene, in a second binding molecule gene, a regulatory gene, or in any other gene which directly or indirectly affects the metabolism or structure of the serine protease or serine protease binding molecule. A mutation is meant to include, e.g., an alteration, e.g., in gross or fine structure, in a nucleic acid. Examples include single base pair alterations, e.g., missense or nonsense mutations, frameshifts, deletions, insertions and translocations. Mutations can be dominant or recessive. Mutations can be homozygous or heterozygous.

An agent is meant to include, e.g., any substance, e.g., an apoptosis-modulating drug. The agent of this invention preferably can change an aspect of the metabolism of the serine protease or the serine protease binding molecule. Such change can be the result of any of a variety of events, including, e.g., preventing, reducing or increasing interaction between one of these polypeptides and a binding molecule; inactivating or activating one of these polypeptides and/or the binding molecule, e.g., by cleavage or other modification; altering the affinity of one of these polypeptides and the binding molecule for each other; diluting out one of these polypeptides and/or a binding molecule; preventing or activating expression of one of these polypeptides and/or a binding molecule; reducing or increasing synthesis of one of these polypeptides and/or a binding molecule; synthesizing an abnormal polypeptide and/or binding molecule; synthesizing an alternatively spliced polypeptide and/or binding molecule; preventing, reducing or increasing proper conformational folding of one of these polypeptides and/or a binding molecule; modulating the binding properties of one of these polypeptides and/or a binding molecule; interfering with or promoting signals that are required to activate or deactivate

one of these polypeptides and/or a binding molecule; activating or deactivating one of these polypeptides and/or a binding molecule at the wrong time; or interfering with other receptors, ligands or other molecules which are required for the normal synthesis or functioning of one of these polypeptides and/or a binding molecule.

Examples of agents include a serine protease or a serine protease binding molecule, or a biologically active fragment or analog thereof; a nucleic acid encoding one of these agents or a biologically active fragment or analog thereof; a nucleic acid encoding a regulatory sequence for one of these agents or a biologically active fragment or analog thereof; a binding molecule for one of these agents; a binding molecule for a nucleic acid encoding one of these agents, the nucleic acid being, e.g., a nucleic acid comprising a regulatory region or a nucleic acid comprising a structural region, or a biologically active fragment thereof; an antisense nucleic acid for one of these agents; a mimetic of one of these agents; an antibody for one of these agents; a metabolite; or an inhibitory carbohydrate or glycoprotein. In certain embodiments, the agent is an antagonist or agonist for the serine protease or serine protease binding molecule. By antagonist is meant a compound which inhibits a function of the agent. By agonist is meant a compound which has similar biological functions as the agent. In certain embodiments, the agent is a natural ligand for the serine protease or the serine protease binding molecule. In certain embodiments, the agent is an artificial ligand for the serine protease or the serine protease binding molecule.

In preferred embodiments, the serine protease agent is a granzyme, preferably granzyme A, or a biologically active fragment or analog thereof, and the serine protease binding molecule agent is a granzyme binding molecule, preferably a granzyme A binding molecule, and more preferably is PHAP I, PHAP II, a complex comprising PHAP II, heat shock protein 27, or a biologically active fragment or analog thereof.

By a nucleic acid encoding an agent, e.g., a polypeptide, is meant a nucleic acid which includes only coding sequence for the polypeptide, as well as a nucleic acid which includes additional coding, e.g., a leader or secretory sequence or a proprotein sequence, and/or non-coding sequences, e.g., introns or non-coding sequences 5' and/or 3' of the coding sequence for the mature polypeptide. The nucleic acid is also meant to include nucleic acids in which the coding sequence for the mature polypeptide is fused in the same reading frame to a nucleic acid sequence which aids in expression and/or secretion of a polypeptide from a host cell, e.g., a leader sequence.

The nucleic acid can be in the form of RNA, DNA or PNA, e.g., cRNA, cDNA, genomic

DNA, or synthetic DNA, RNA or PNA. The DNA can be double stranded or single stranded, and if single stranded can be the coding strand or non-coding (anti-sense) strand. The coding sequence which encodes a polypeptide can be identical to the naturally-occurring gene, or can be different as a result of the redundancy or degeneracy of the genetic code.

By analog is meant a compound that differs from the naturally occurring serine protease or the serine protease binding molecule in amino acid sequence or in ways that do not involve sequence, or both. Analogs of the invention generally exhibit at least about 80% homology, preferably at least about 90% homology, more preferably yet at least about 95% homology, and most preferably at least about 98% homology, with substantially the entire sequence of a naturally occurring serine protease or the serine protease binding molecule sequence, preferably with a segment of about 100 amino acid residues, more preferably with a segment of about 50 amino acid residues, more preferably yet with a segment of about 30 amino acid residues, more preferably yet with a segment of about 20 amino acid residues, more preferably yet with a segment of about 10 amino acid residues, and most preferably yet with a segment of about 5 amino acid residues. Non-sequence modifications include, e.g., in vivo or in vitro chemical derivatizations of the serine protease or the serine protease binding molecule. Non-sequence modifications include, e.g., changes in phosphorylation, acetylation, methylation, carboxylation, or glycosylation. Methods for making such modifications are known to those skilled in the art. For example, phosphorylation can be modified by exposing the polypeptide to phosphorylation-altering enzymes, e.g., kinases or phosphatases.

Preferred analogs include a serine protease or serine protease binding molecule, or biologically active fragments thereof, whose sequences differ from the wild-type sequence by one or more conservative amino acid substitutions or by one or more non-conservative amino acid substitutions, deletions, or insertions which do not abolish biological activity of the polypeptide. Conservative substitutions typically include the substitution of one amino acid for another with similar characteristics, e.g., substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. Other examples of conservative substitutions are shown in Table 1.

Table 1

CONSERVATIVE AMINO ACID SUBSTITUTIONS

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly, beta-Ala, L-Cys, D-Cys
Arginine	R	D-Arg, Lys, D-Lys, homo-Arg, D-homo-Arg, Met, Ile, D-Met, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn, Asp, D-Asp, Glu, D-Glu, Gln, D-Gln
Aspartic Acid	D	D-Asp, D-Asn, Asn, Glu, D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys, Met, D-Met, Thr, D-Thr
Glutamine	Q	D-Gln, Asn, D-Asn, Glu, D-Glu, Asp, D-Asp
Glutamic Acid	E	D-Glu, D-Asp, Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala, Pro, D-Pro, β-Ala Acp
Histidine	H	D-His
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met
Leucine	L	D-Leu, Val, D-Val, Leu, D-Leu, Met, D-Met
Lysine	K	D-Lys, Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val
Phenylalanine	F	D-Phe, Tyr, D-Thr, L-Dopa, His, D-His, Trp, D-Trp, Trans-3,4, or 5-phenylproline, cis-3,4, or 5-phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D-or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), L-Cys, D-Cys
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Tryptophan	W	D-Trp, Phe, D-Phe, Tyr, D-Tyr
Tyrosine	Y	D-Tyr, Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu, D-Leu, Ile, D-Ile, Met, D-Met

Amino acid sequence variants of a protein can be prepared by any of a variety of methods known to those skilled in the art. For example, random mutagenesis of DNA which encodes a protein or a particular domain or region of a protein can be used, e.g., PCR mutagenesis (using, e.g., reduced *Taq* polymerase fidelity to introduce random mutations into a cloned fragment of DNA; Leung et al., BioTechnique 1:11-15 (1989)), or saturation mutagenesis (by, e.g., chemical treatment or irradiation of single-stranded DNA in vitro, and synthesis of a complementary DNA strand; Mayers et al., Science 229:242 (1985)). Random mutagenesis can also be accomplished by, e.g., degenerate oligonucleotide generation (using, e.g., an automatic DNA synthesizer to chemically synthesize degenerate sequences; Narang, Tetrahedron 39:3 (1983); Itakura et al.,

Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules, ed. A.G. Walton, Amsterdam: Elsevier, pp. 273-289 (1981)). Non-random or directed mutagenesis can be used to provide specific sequences or mutations in specific regions. These techniques can be used to create variants which include, e.g., deletions, insertions, or substitutions, of residues of the known amino acid sequence of a protein. The sites for mutation can be modified individually or in series, e.g., by (i) substituting first with conserved amino acids and then with more radical choices depending upon results achieved, (ii) deleting the target residue, (iii) inserting residues of the same or a different class adjacent to the located site, or (iv) combinations of the above.

Methods for identifying desirable mutations include, e.g., alanine scanning mutagenesis (Cunningham and Wells, Science 244:1081-1085 (1989)), oligonucleotide-mediated mutagenesis (Adelman et al., DNA 2:183 (1983)); cassette mutagenesis (Wells et al., Gene 34:315 (1985)), combinatorial mutagenesis, and phage display libraries (Ladner et al., PCT International Appln. No. WO88/06630). The serine protease or the serine protease binding molecule analogs can be tested, e.g., for their ability to bind to a binding molecule, e.g., as described herein.

Other analogs within the invention include, e.g., those with modifications which increase peptide stability. Such analogs may contain, e.g., one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are, e.g.: analogs that include residues other than naturally occurring L-amino acids, e.g., D-amino acids or non-naturally occurring or synthetic amino acids, e.g., β or γ amino acids; and cyclic analogs.

Analogs are also meant to include peptides in which structural modifications have been introduced into the peptide backbone so as to make the peptide non-hydrolyzable. Such peptides are particularly useful for oral administration, as they are not digested. Peptide backbone modifications include, e.g., modifications of the amide nitrogen, the α-carbon, the amide carbonyl, or the amide bond, and modifications involving extensions, deletions or backbone crosslinks. For example, the backbone can be modified by substitution of a sulfoxide for the carbonyl, by reversing the peptide bond, or by substituting a methylene for the carbonyl group. Such modifications can be made by standard procedures known to those skilled in the art. See, e.g., Spatola, A.F., "Peptide Backbone Modifications: A Structure-Activity Analysis of Peptides Containing Amide Bond Surrogates, Conformational Constraints, and Related Backbone Replacements," in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp. 267-357, B. Weinstein (ed.), Marcel Dekker, Inc., New York (1983).

An analog is also meant to include polypeptides in which one or more of the amino acid residues include a substituent group, or polypeptides which are fused with another compound,

e.g., a compound to increase the half-life of the polypeptide, e.g., polyethylene glycol.

By fragment is meant some portion of the naturally occurring serine protease or serine protease binding molecule polypeptide. Preferably, the fragment is at least about 100 amino acid residues, more preferably at least about 50 amino acid residues, more preferably yet at least about 30 amino acid residues, more preferably yet at least about 20 amino acid residues, and most preferably at least about 5 amino acid residues in length. Fragments include, e.g., truncated secreted forms, cleaved fragments, proteolytic fragments, splicing fragments, other fragments, and chimeric constructs between at least a portion of the relevant gene and another molecule. Fragments of a serine protease or the serine protease binding molecule can be generated by methods known to those skilled in the art. In certain embodiments, the fragment is biologically active. The ability of a candidate fragment to exhibit a biological activity of the serine protease or serine protease binding molecule can be assessed by methods known to those skilled in the art, e.g., as described herein. Also included are fragments containing residues that are not required for biological activity of the fragment or that result from alternative mRNA splicing or alternative protein processing events.

Fragments of a protein can be produced by any of a variety of methods known to those skilled in the art, e.g., recombinantly, by proteolytic digestion, or by chemical synthesis. Internal or terminal fragments of a polypeptide can be generated by removing one or more nucleotides from one end (for a terminal fragment) or both ends (for an internal fragment) of a nucleic acid which encodes the polypeptide. Expression of the mutagenized DNA produces polypeptide fragments. Digestion with "end-nibbling" endonucleases can thus generate DNAs which encode an array of fragments. DNAs which encode fragments of a protein can also be generated, e.g., by random shearing, restriction digestion or a combination of the above-discussed methods.

Fragments can also be chemically synthesized using techniques known in the art, e.g., conventional Merrifield solid phase f-Moc or t-Boc chemistry. For example, peptides of the present invention can be arbitrarily divided into fragments of desired length with no overlap of the fragments, or divided into overlapping fragments of a desired length.

A serine protease or a biologically active fragment or analog thereof, or a serine protease binding molecule or a biologically active fragment or analog thereof, can, e.g., compete with its cognate molecule for the binding site on the complementary molecule, and thereby reduce or eliminate binding between the serine protease or the serine protease binding molecule and a cellular binding molecule. The serine protease or the serine protease binding molecule can be obtained, e.g., from purification or secretion of naturally occurring serine protease or serine

protease binding molecule, from recombinant serine protease or serine protease binding molecule, or from synthesized serine protease or serine protease binding molecule.

Therefore, methods for generating analogs and fragments and testing them for activity are known to those skilled in the art.

An agent can also be a nucleic acid used as an antisense molecule. Antisense therapy is meant to include, e.g., administration or in situ generation of oligonucleotides or their derivatives which specifically hybridize, e.g., bind, under cellular conditions, with the cellular mRNA and/or genomic DNA encoding a serine protease or the serine protease binding molecule, or mutants thereof, so as to inhibit expression of the encoded protein, e.g., by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific interactions in the major or minor groove of the double helix.

In certain embodiments, the antisense construct binds to a naturally-occurring sequence of a serine protease gene or a serine protease binding molecule gene, which, e.g., is involved in expression of the gene. These sequences include, e.g., promoters, start codons, stop codons, and RNA polymerase binding sites.

In other embodiments, the antisense construct binds to a nucleotide sequence which is not present in the wild type gene. For example, the antisense construct can bind to a region of a serine protease gene or serine protease binding molecule gene which contains an insertion of an exogenous, non-wild type sequence. Alternatively, the antisense construct can bind to a region of one of these genes which has undergone a deletion, thereby bringing two regions of the gene together which are not normally positioned together and which, together, create a non-wild type sequence. When administered in vivo to a subject, antisense constructs which bind to non-wild type sequences provide the advantage of inhibiting the expression of a mutant serine protease gene or serine protease binding molecule gene, without inhibiting expression of the wild type gene.

In preferred embodiments, the antisense nucleic acid is for granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, heat shock protein 27, or a biologically active fragment or analog thereof.

An antisense construct of the present invention can be delivered, e.g., as an expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a serine protease or a serine protease binding molecule. An alternative is that the antisense construct is an oligonucleotide which is

generated ex vivo and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA (duplexing) and/or genomic sequences (triplexing) of a serine protease or serine protease binding molecule gene. Such oligonucleotides are preferably modified oligonucleotides which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and are therefore stable in vivo. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothiorate and methylphosphonate analogs of DNA and peptide nucleic acids (PNA). (See also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to constructing oligomers useful in antisense therapy have been reviewed. (See, e.g., Van der Krol et al., Biotechniques 6:958-976, (1988); Stein et al., Cancer Res. 48:2659-2668 (1988)).

By mimetic is meant a molecule which resembles in shape and/or charge distribution a serine protease or a serine protease binding molecule. Preferably, the mimetic is of granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, heat shock protein 27, or a biologically active fragment or analog thereof. The mimetic can be a peptide or a non-peptide. Mimetics can act as therapeutic agents because they can, e.g., competitively inhibit binding of a serine protease to a binding molecule, or a serine protease binding molecule to another binding molecule. By employing, e.g., scanning mutagenesis, e.g., alanine scanning mutagenesis, linker scanning mutagenesis or saturation mutagenesis, to map the amino acid residues of a particular serine protease or the serine protease binding molecule polypeptide involved in binding a binding molecule, peptide mimetics, e.g., diazepine or isoquinoline derivatives, can be generated which mimic those residues in binding to a binding molecule, and which therefore can inhibit binding of the serine protease or the serine protease binding molecule to a binding molecule and thereby interfere with the function of the serine protease or the serine protease binding molecule. Non-hydrolyzable peptide analogs of such residues can be generated using, e.g., benzodiazepine (see, e.g., Freidinger et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands (1988)); azepine (see, e.g., Huffman et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands (1988)); substituted gamma lactam rings (see, e.g., Garvey et al., in Peptides: Chemistry and Biology, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands (1988)); keto-methylene pseudopeptides (see, e.g., Ewenson et al., J. Med. Chem. 29:295 (1986); Ewenson et al., in Peptides: Structure and Function (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL (1985)); β-turn dipeptide cores (see, e.g., Nagai et al., Tetrahedron Lett. 26:647 (1985); Sato et al., J. Chem. Soc. Perkin Trans. 1:1231 (1986)); or β-aminoalcohols (see, e.g., Gordon et al.,

Biochem. Biophys. Res. Commun. 126:419 (1985); Dann et al., Biochem. Biophys. Res. Commun. 134:71 (1986)).

Antibodies are meant to include antibodies against any moiety that directly or indirectly affects the metabolism of a serine protease or a serine protease binding molecule, preferably granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27. The antibodies can be directed against, e.g., a serine protease or a serine protease binding molecule, or a complex, subunit, fragment or analog thereof, or a biologically active fragment or analog thereof. For example, antibodies include anti-granzyme A, anti-PHAP I, anti-PHAP II, anti-PHAP II-containing complex, and anti-heat shock protein 27 antibodies. Antibody fragments are meant to include, e.g., Fab fragments, Fab' fragments, $F(ab')_2$ fragments, F(v) fragments, heavy chain monomers, heavy chain dimers, heavy chain trimers, light chain monomers, light chain dimers, light chain trimers, dimers consisting of one heavy and one light chain, and peptides that mimic the activity of the anti-serine protease or anti-serine protease binding molecule antibodies. For example, Fab_2' fragments of the inhibitory antibody can be generated through, e.g., enzymatic cleavage.

Both polyclonal and monoclonal antibodies can be used in this invention. Preferably, monoclonal antibodies are used. Natural antibodies, recombinant antibodies or chimeric-antibodies, e.g., humanized antibodies, are included in this invention. Preferably, humanized antibodies are used when the subject is a human. Most preferably, the antibodies have a constant region derived from a human antibody and a variable region derived from an inhibitory mouse monoclonal antibody. Monoclonal and humanized antibodies are generated by standard methods known to those skilled in the art. Monoclonal antibodies can be produced, e.g., by any technique which provides antibodies produced by continuous cell lines cultures. Examples include the hybridoma technique (Kohler and Milstein, Nature 256:495-497 (1975), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology Today 4:72 (1983)), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., in Monoclonal Antibodies and Cancer Therapy, A.R. Liss, Inc., pp. 77-96 (1985)). Preferably, humanized antibodies are raised through conventional production and harvesting techniques (Berkower, I., Curr. Opin. Biotechnol. 7:622-628 (1996); Ramharayan and Skaletsky, Am. Biotechnol. Lab 13:26-28 (1995)). For example, in certain preferred embodiments, the antibodies are raised against the serine protease, preferably against a binding site for a serine protease binding molecule, and the Fab fragments produced. These antibodies, or fragments derived therefrom, can be used, e.g., to block the serine protease binding molecule binding sites on the serine

protease molecules. Production of monoclonal and polyclonal antibodies are described in Examples 3 and 11.

Agents also include inhibitors of a molecule that are required for synthesis, post-translational modification, or functioning of a serine protease or serine protease binding molecule, or activators of a molecule that inhibits the synthesis or functioning of a serine protease or a serine protease binding molecule. Agents include, e.g., cytokines, chemokines, growth factors, hormones, signaling components, kinases, phosphatases, homeobox proteins, transcription factors, editing factors, translation factors and post-translation factors or enzymes. Agents are also meant to include ionizing radiation, non-ionizing radiation, ultrasound and toxic agents which can, e.g., at least partially inactivate or destroy the serine protease or the serine protease binding molecule.

An agent is also meant to include an agent which is not entirely serine protease or serine protease binding molecule specific. For example, an agent may alter other genes or proteins related to apoptosis. Such overlapping specificity may provide additional therapeutic advantage.

The invention also includes the agent so identified as being useful in modulating apoptosis.

The invention also includes a method for effecting apoptosis in a cell. A cell which is deficient in effecting apoptosis is provided. An effective amount of an active endogenous DNase capable of degrading genomic DNA in the cell is provided. The DNase is administered to the cell so as to effect apoptosis.

By being deficient in effecting apoptosis is meant, e.g., being partially or completely deficient. The deficiency can result from any cause which can be overcome by the provision of active endogenous DNase such that the DNase can degrade genomic DNA in the cell. For example, there might not be enough DNase in the cell, there might not be enough active DNase in the cell, e.g., in an active cleaved or processed form, or there might not be enough active DNase in the proper compartment in the cell. In certain embodiments, the endogenous DNase is a binding molecule for a serine protease, e.g., a granzyme, e.g., granzyme A, or is a binding molecule for a serine protease binding molecule, e.g., a granzyme binding molecule, e.g., a granzyme A binding molecule, or is part of a complex that comprises a binding molecule for a serine protease. In certain embodiments, the endogenous DNase is a complex comprising PHAP II. In certain embodiments, the endogenous DNase is a component of a complex comprising PHAP II.

In certain embodiments, the effective amount of active endogenous DNase is provided by

providing PHAP II or a complex comprising PHAP II, or an active analog or fragment thereof, to the cell. An alternative is providing to the cell a nucleic acid encoding PHAP II or a component of a complex comprising PHAP II, or an active analog or fragment thereof, and expressing PHAP II or the component of the complex comprising PHAP II, or the active analog or fragment thereof in vivo. In certain embodiments, the effective amount of active endogenous DNase is provided by providing granzyme A or an active analog or fragment thereof to the cell so as to cleave PHAP II. An alternative is providing a nucleic acid encoding granzyme A or an active analog or fragment thereof and expressing granzyme A or the active analog or fragment thereof in vivo. In yet other embodiments, the effective amount of active endogenous DNase is provided by providing heat shock protein 27 or an active analog or fragment thereof to the cell. An alternative is providing a nucleic acid encoding heat shock protein 27 or an active analog or fragment thereof and expressing heat shock protein 27 or the active analog or fragment thereof in vivo.

The invention also includes a method for inhibiting apoptosis in a cell. A cell which is capable of effecting apoptosis is provided. An agent which inhibits genomic DNA degradation in the cell by an endogenous DNase is provided. The agent is administered to the cell so as to inhibit apoptosis in the cell.

In certain embodiments, the endogenous DNase is a binding molecule for a serine protease, e.g., a granzyme, e.g., granzyme A, or is a binding molecule for a serine protease binding molecule, e.g., a granzyme binding molecule, e.g., a granzyme A binding molecule, or is part of a complex that comprises a binding molecule for a serine protease. In certain embodiments, the endogenous DNase is a complex comprising PHAP II. In certain embodiments, the endogenous DNase is a component of a complex comprising PHAP II.

The invention also includes a method for treating unwanted cell or infectious particle proliferation in an animal. An animal having cells in need of treatment for unwanted cell or infectious particle proliferation is provided. An agent capable of activating apoptosis is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the unwanted cell or infectious particle proliferation in the animal occurs.

Unwanted cell proliferation includes, e.g., a malignancy or cancer, e.g., leukemia, lymphoma, breast cancer, melanoma, renal cell carcinoma, or lymphoproliferation in, e.g., graft versus host disease, autoimmune disease or graft rejection. By graft versus host disease is meant that the lymphocytes in the donor graft inappropriately attack and kill host tissue cells. Unwanted cell proliferation can also result from, e.g., surgical scarring or restenosis. Unwanted

infectious particle proliferation includes, e.g., growth of infectious viruses, bacteria or other microorganisms in the animal, e.g., from pathogenic infectious particle infection, e.g., HIV, hepatitis B or C, EBV, CMV, mycobacteria, Candida or Pneumocystis pneumoniae.

In preferred embodiments, the apoptosis that is activated is mediated by a granzyme, preferably granzyme A. The agent can be a polypeptide or any other type of molecule. In certain preferred embodiments, the agent is a serine protease or a binding molecule for a serine protease, or biologically active analogs or fragments thereof. Preferably, the serine protease is a granzyme, e.g., granzyme A. Preferably, the binding molecule is a granzyme binding molecule, preferably a granzyme A binding molecule, and more preferably is PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27. In certain embodiments, the therapeutically effective amount of the polypeptide is administered by providing to the animal a nucleic acid encoding the polypeptide, e.g., a serine protease or a serine protease binding molecule, or biologically active analogs or fragments thereof, and expressing the polypeptide in vivo.

In certain embodiments, the nucleic acid is administered to the animal by introducing the nucleic acid into the cells of the animal ex vivo and administering the cells having the nucleic acid to the animal. In yet other embodiments, the agent is a mimetic of a serine protease, of a binding molecule for a serine protease, or of biologically active analogs or fragments thereof. In certain embodiments, the agent is an agonist of a serine protease or an agonist of a serine protease binding molecule.

Treating is meant to include, e.g., preventing, treating, reducing the symptoms of, or curing the disease. Administration of the agent can be accomplished by any method which allows the agent to reach the target cells. These methods include, e.g., injection, deposition, implantation, suppositories, oral ingestion, inhalation, topical administration, or any other method of administration where access to the target cells by the agent is obtained. Injections can be, e.g., intravenous, intradermal, subcutaneous, intramuscular or intraperitoneal. Implantation includes inserting implantable drug delivery systems, e.g., microspheres, hydrogels, polymeric reservoirs, cholesterol matrices, polymeric systems, e.g., matrix erosion and/or diffusion systems and non-polymeric systems, e.g., compressed, fused or partially fused pellets. Suppositories include glycerin suppositories. Oral ingestion doses can be enterically coated. Inhalation includes administering the agent with an aerosol in an inhalator, either alone or attached to a carrier that can be absorbed.

In certain embodiments, the agent is targeted to one or more cell types, or to one or more cells within a cell type, so as to be therapeutically effective, by methods known to those skilled

in the art. For example, the agent can be coupled to an antibody, to a ligand to a cell surface receptor, or to a toxin component, or can be contained in a particle which is selectively internalized into cells, e.g., liposomes, or a virus where the viral receptor binds specifically to a certain cell type, or a viral particle lacking the viral nucleic acid, or can be administered by local injection.

Administration of the agent can be alone or in combination with other therapeutic agents. In certain embodiments, the agent can be combined with a suitable carrier, incorporated into a liposome, or incorporated into a polymer release system.

In certain embodiments of the invention, the administration can be designed so as to result in sequential exposures to the agent over some time period, e.g., hours, days, weeks, months or years. This can be accomplished by repeated administrations of the agent by one of the methods described above, or alternatively, by a controlled release delivery system in which the agent is delivered to the animal over a prolonged period without repeated administrations. By a controlled release delivery system is meant that total release of the agent does not occur immediately upon administration, but rather is delayed for some time. Release can occur in bursts or it can occur gradually and continuously. Administration of such a system can be, e.g., by long acting oral dosage forms, bolus injections, transdermal patches or subcutaneous implants.

Examples of systems in which release occurs in bursts include, e.g., systems in which the agent is entrapped in liposomes which are encapsulated in a polymer matrix, the liposomes being sensitive to a specific stimulus, e.g., temperature, pH, light, magnetic field, or a degrading enzyme, and systems in which the agent is encapsulated by an ionically-coated microcapsule with a microcapsule core-degrading enzyme. Examples of systems in which release of the agent is gradual and continuous include, e.g., erosional systems in which the agent is contained in a form within a matrix, and diffusional systems in which the agent permeates at a controlled rate, e.g., through a polymer. Such sustained release systems can be, e.g., in the form of pellets or capsules.

The agent can be suspended in a liquid, e.g., in dissolved form or colloidal form. The liquid can be a solvent, partial solvent or non-solvent. In many cases water or an organic liquid can be used.

The agent can be administered prior to or subsequent to the appearance of disease symptoms. In certain embodiments, the agent is administered to patients with familial histories of the disease, or who have phenotypes that may indicate a predisposition to the disease, or who

have been diagnosed as having a genotype which predisposes the patient to the disease, or who have other risk factors.

The agent is administered to the animal in a therapeutically effective amount. By therapeutically effective amount is meant that amount which is capable of at least partially preventing or reversing the disease. A therapeutically effective amount can be determined on an individual basis and will be based, at least in part, on consideration of the species of animal, the animal's size, the animal's age, the agent used, the type of delivery system used, the time of administration relative to the onset of disease symptoms, and whether a single, multiple, or controlled release dose regimen is employed. A therapeutically effective amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

Preferably, the concentration of the agent is at a dose of about 0.1 to about 1000 mg/kg body weight/day, more preferably at about 0.1 to about 500 mg/kg/day, more preferably yet at about 0.1 to about 100 mg/kg/day, and most preferably at about 0.1 to about 5 mg/kg/day. The specific concentration partially depends upon the particular agent used, as some are more effective than others. The dosage concentration of the agent that is actually administered is dependent at least in part upon the final concentration that is desired at the site of action, the method of administration, the efficacy of the particular agent, the longevity of the particular agent, and the timing of administration relative to the onset of the disease symptoms. Preferably, the dosage form is such that it does not substantially deleteriously affect the animal. The dosage can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

In certain embodiments, a therapeutically effective amount of an agent which is a polypeptide can be administered by providing to the animal a nucleic acid encoding the polypeptide and expressing the polypeptide in vivo. Various gene constructs can be used as part of a gene therapy protocol to deliver nucleic acids encoding an agent, e.g., either an agonistic or antagonistic form of a polypeptide, e.g., a serine protease or a serine protease binding molecule. For example, expression vectors can be used for in vivo transfection and expression of a polypeptide in particular cell types so as to reconstitute the function of, or alternatively, abrogate the function of, the polypeptide in a cell in which non-wild type polypeptide is expressed. Expression constructs of the polypeptide, and mutants thereof, can be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively delivering the gene for the agent to cells in vivo. Approaches include, e.g., insertion of the

subject gene in viral vectors including, e.g., recombinant retroviruses, adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors infect or transduce cells directly. In certain preferred embodiments, the virus is administered by injection, e.g., intramuscular injection, in a dose range of about 10^3 to about 10^6 infectious particles per injection, more preferably in a dose range of about 10^5 to about 10^8 infectious particles per injection. Single or multiple doses can be administered over a given period of time, depending, e.g., upon the disease. Plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin™ (Life Technologies, Inc., Gaithersburg, MD) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramacidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or $Ca_3(PO_4)_2$ precipitation carried out in vivo, or by use of a gene gun. The above-described methods are known to those skilled in the art and can be performed without undue experimentation. Since transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g., locally or systemically. Administration can be directed to one or more cell types, and to one or more cells within a cell type, so as to be therapeutically effective, by methods known to those skilled in the art. In certain embodiments, administration is done in a prenatal animal or embryonic cell. It will be recognized that the particular gene construct provided for in in vivo transduction of the agent is also useful for in vitro transduction of cells, such as for use in diagnostic assays.

In certain embodiments, the nucleic acid is administered to the animal by introducing the nucleic acid into cells of the animal ex vivo and administering the cells having the nucleic acid to the animal. Preferably, the cells are administered in a dose range of about 1×10^6 to about 1×10^9 cells/dosage/day, and most preferably at about 1×10^7 to about 1×10^8 cells/dosage/day.

In certain embodiments, other therapy is additionally administered. For example, if the animal is being treated for a tumor, other tumor therapy, e.g., chemotherapy, radiation or surgery, is additionally administered to the animal.

The invention also includes a method for providing an animal having cancer with a therapeutic level of a polypeptide, e.g., a granzyme, e.g., granzyme A, a binding molecule for a granzyme, e.g., a binding molecule for granzyme A, e.g., PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27, or a biologically active analog or fragment thereof. The polypeptide is provided to the animal, e.g., by administering to the animal the polypeptide itself or by administering to the animal a nucleic acid encoding the polypeptide.

The invention also includes a method for treating an animal at risk for unwanted cell or infectious particle proliferation. An animal at risk for unwanted cell or infectious particle proliferation is provided. An agent capable of activating apoptosis is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the unwanted cell or infectious particle proliferation occurs. Being at risk for the disease can result from, e.g., a family history of the disease, a genotype which predisposes to the disease, or phenotypic symptoms which predispose to the disease.

The invention also includes a pharmaceutical composition for treating unwanted cell or infectious particle proliferation in an animal comprising a therapeutically effective amount of an agent capable of effecting apoptosis, e.g., a serine protease, a binding molecule for a serine protease, or a biologically active analog or fragment thereof, and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers include, e.g., water, saline, dextrose, glycerol, ethanol, liposomes and lipid emulsions. In preferred embodiments, the serine protease is a granzyme, e.g., granzyme A, and the binding molecule for a serine protease is a granzyme binding molecule, e.g., a granzyme A binding molecule, e.g., PHAP I, PHAP II, a complex comprising PHAP II or heat shock protein 27.

The invention also includes a pharmaceutical composition for treating unwanted cell or infectious particle proliferation in an animal comprising a therapeutically effective amount of a recombinant nucleic acid encoding a polypeptide capable of effecting apoptosis, e.g., a serine protease, a binding molecule for a serine protease, or a biologically active analog or fragment thereof, and a pharmaceutically acceptable carrier.

The invention also includes a method for treating an autoimmune disease or a transplant graft rejection in an animal. An animal in need of treatment for an autoimmune disease or a transplant graft rejection is provided. An agent which inhibits apoptosis is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the autoimmune disease or transplant graft rejection occurs.

By autoimmune disease is meant injury resulting from the inappropriate targeting by the immune system of self-components. Such autoimmune diseases can be treated by inhibiting apoptosis. Examples of autoimmune diseases that can be treated by this invention include rheumatoid arthritis, juvenile rheumatoid arthritis, fasciitis (excluding eosinophilic fasciitis), polymyositis, dermatomyositis, panniculitis, ankylosing spondylitis, Reiter's syndrome, necrotizing vasculitis (including polyarteriitis nodosa), psoriatic arthritis, inflammatory bowel disease, psoriasis (especially psoriatic arthritis), juvenile diabetes, systemic lupus erythematosus,

scleroderma, granulomatous diseases (including, e.g., sarcoid, Wegener's granulomatosis, Takayasu's arteriitis and giant cell arteriitis).

By transplant graft is meant cells or tissue taken from an animal induced to grow or differentiate and function in a second animal. A transplant graft rejection can be associated with programmed cell death of the transplanted cells or tissue induced by activation of immune T cells. Transplant graft rejection can occur, e.g., as a result of tissue or organ transplants. Preferably, administration is accomplished by introducing the agent into the graft itself. Administration can be before or after transplantation.

In preferred embodiments, the apoptosis that is inhibited by the agent is mediated by a granzyme, preferably granzyme A. Preferably, the agent is an inhibitor of a serine protease, e.g., a granzyme, e.g., granzyme A, or a serine protease binding molecule, e.g., a granzyme binding molecule, e.g., a granzyme A binding molecule, e.g., PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27. In certain embodiments, the agent is an antagonist of a serine protease or a serine protease binding molecule. In certain embodiments, the agent is a polyclonal or monoclonal antibody, or a fragment thereof, that can immunoreact with granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27. In certain embodiments, the therapeutically effective amount of the agent is administered by providing the agent itself to the animal. In certain embodiments, the therapeutically effective amount of the agent is administered by providing to the animal a nucleic acid encoding the agent and expressing the agent in vivo.

The invention also includes a method for treating an autoimmune disease or a transplant graft rejection in an animal. A therapeutically effective amount of an agent capable of inhibiting DNase activity of a complex comprising PHAP II or a component of the complex, or capable of inhibiting granzyme A cleavage of PHAP II, is administered to an animal having an autoimmune disease or a transplant graft rejection.

The invention also includes a method for treating an animal at risk for an autoimmune disease or a transplant graft rejection. An animal at risk for an autoimmune disease or a transplant graft rejection is provided. An agent capable of inhibiting apoptosis is provided. The agent is administered to the animal in a therapeutically effective amount such that treatment of the autoimmune disease or transplant graft rejection occurs.

The invention also includes a pharmaceutical composition for treating an autoimmune disease or a tissue graft rejection in an animal comprising a therapeutically effective amount of an agent capable of inhibiting apoptosis by inhibiting a serine protease, a binding molecule for a

serine protease, or a biologically active analog or fragment thereof, and a pharmaceutically acceptable carrier. In preferred embodiments, the serine protease is a granzyme, e.g., granzyme A, and the binding molecule for a serine protease is a granzyme binding molecule, e.g., a granzyme A binding molecule, e.g., PHAP I, PHAP II, a complex comprising PHAP II or heat shock protein 27.

The invention also includes a pharmaceutical composition for treating an autoimmune disease or a transplant graft rejection in an animal comprising a therapeutically effective amount of a recombinant nucleic acid encoding a polypeptide capable of inhibiting apoptosis by inhibiting a serine protease, a binding molecule for a serine protease, or a biologically active analog or fragment thereof, and a pharmaceutically acceptable carrier.

The invention also includes a pharmaceutical composition for treating an autoimmune disease or a transplant graft rejection in an animal comprising a therapeutically effective amount of cells wherein a recombinant nucleic acid encoding a polypeptide capable of inhibiting apoptosis by inhibiting a serine protease, a binding molecule of a serine protease or a biologically active analog or fragment thereof, has been introduced into the cells so as to express the polypeptide, and a pharmaceutically acceptable carrier.

The invention also includes a method of degrading nucleic acid in vitro. Nucleic acid is provided. An isolated complex comprising PHAP II is provided. The nucleic acid is contacted with the complex in vitro such that the nucleic acid is degraded by the complex comprising PHAP II. The nucleic acid can be, e.g., DNA or RNA. In certain embodiments, the nucleic acid is provided in an infectious particle.

The invention also includes a method of degrading nucleic acid in vivo. A cell having nucleic acid that is deficient in active endogenous DNase is provided. A complex comprising PHAP II is provided. The nucleic acid is contacted with the complex in vivo such that the nucleic acid is degraded by the complex. In preferred embodiments, the complex comprising PHAP II that is provided is an isolated complex. In preferred embodiments, the active endogenous DNase that is deficient in the cell is an active endogenous DNase for apoptosis. In certain embodiments, the complex can be provided, e.g., by providing PHAP II, granzyme A, or heat shock protein 27, or active analogs or fragments thereof. The nucleic acid can be, e.g., DNA or RNA. In certain embodiments, the complex can be provided by, e.g., adding a nucleic acid encoding PHAP II, granzyme A or heat shock protein 27, or an active analog or fragment thereof.

The invention also includes a method for identifying an enzyme substrate. An enzyme

having an inactive active site is provided. A composition having a candidate substrate for the enzyme is provided. The composition is contacted wtih the enzyme and it is determined if the candidate substrate in the composition binds to the enzyme so as to identify the candidate substrate as a substrate for the enzyme.

Any method known by those skilled in the art for determining binding can be used, e.g., affinity chromatography, coprecipitation or coimmunoprecipitation. Preferably, affinity chromatography is used. In certain embodiments, the enzyme is coupled to a matrix, e.g., a matrix for affinity chromatography.

Enzyme is meant to include, e.g., a natural enzyme, a recombinant enzyme or a chemically synthesized enzyme. The enzyme can have an inactive active site as a result of, e.g., a mutation. In certain embodiments, the enzyme is a serine protease, e.g., a granzyme. See Example 6 in which recombinant inactive granzyme A with active site S→A was used to isolate, e.g., the substrate PHAP II.

The invention also includes a recombinant expression vector comprising a nucleic acid encoding granzyme A, a leader sequence for periplasmic export of the granzyme A, and a carboxy terminal tag for purification of the granzyme A. In certain embodiments, the vector also has an enterokinase site 5' of the nucleic acid encoding the granzyme A so as to enable in vitro activation of the granzyme A.

The granzyme A can be from any animal, including human. The vector can be, e.g., a plasmid, a viral particle or a phage. Any vector can be used, e.g., chromosomal, non-chromosomal or synthetic vectors. In preferred embodiments, the recombinant expression vector is a bacterial recombinant expression vector, e.g., an Escherichia coli recombinant expression vector. Other vectors include, e.g,. Baculovirus or vaccinia virus. The leader sequence can be any leader sequence known to those skilled in the art, e.g., a pel B leader sequence. The N or carboxy terminal tag can be any tag known to those skilled in the art, e.g., a $(His)_6$ tag or a FLAG epitope antibody. An advantage of the vectors of this invention is, e.g., that a soluble granzyme A precursor can be produced in E. coli and cleaved in vitro with enterokinase to an active protease, e.g., rGranA. The invention also includes recombinant expression vectors having an inactive granzyme, e.g., an inactive granzyme can be produced by mutating the active site Ser to Ala, S->ArGranA, as described herein. Such an inactive granzyme can be used to identify binding molecules for granzyme A, as described herein.

The invention also includes a cell having a recombinant expression vector described above. The recombinant vectors described herein can be introduced into a cell, e.g., by

transformation, transfection, transduction, infection or ex vivo injection. Preferably, they are targeted to a particular cell type or cell.

The invention also includes a method for producing granzyme A comprising culturing a cell having a recombinant expression vector described above under conditions that permit expression of the granzyme A.

The invention also includes recombinant expression vectors for PHAP I, PHAP II, and heat shock protein 27.

The following non-limiting examples further illustrate the present invention.

EXAMPLES

Example 1: Production of Active Recombinant Human Granzyme A (rGranA) in E. coli

This example illustrates construction of a granzyme A bacterial expression plasmid which contains a pel B leader sequence for periplasmic export, an enterokinase site immediately 5' of the active coding sequence to enable in vitro enzyme activation, and a carboxy terminal $(His)_6$ tag for purification. See Fig. 1.

Human granzyme A cDNA was PCR amplified with Vent polymerase (New England Biolabs, Beverly, MA) from reverse transcribed mRNA isolated from mitogen activated peripheral blood cells. (Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). Primers were constructed from the human granzyme A sequence (Gershenfeld and Weissman, Science 232:854 (1986)) to contain BamH1 and EcoR1 restriction sites. The PCR product was directionally ligated into pGBT9 (Clontech, Palo Alto, CA). The granzyme insertion was excised and modified by PCR amplification with primers encoding an enterokinase site 5' of the predicted first amino acid of the active enzyme and BamH1 and Xho1 restriction sites for insertion into pet26b (Novagen, Madison, WI). The sequence was verified via dideoxy sequencing (Sequenase; USB/Amersham, Cleveland, OH). Plasmid expression of transfected colonies of BL21-DE3 (Novagen, Madison, WI) was induced with 1 mM IPTG. Bacterial pellets, resuspended in nickel column binding buffer (5 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9) with 0.1% NP40 (Sigma, St. Louis, MO), were sonicated and centrifuged for 20 minutes at 40,000 xg. RGranA was eluted with 125 mM imidazole elution buffer from Novagen nickel resin using FPLC (Biologic Chromatography system; Bio Rad, Hercules, CA). Fractions containing granzyme A were pooled and desalted over a HITRAP column (Pharmacia, Piscataway, NJ) into enterokinase buffer (20 mM Tris-HCl, 50 mM NaCl, 2 mM $CaCl_2$) and digested with ten units of porcine

enterokinase (Sigma) at 21 °C for 12 hours, desalted into nickel binding buffer and repurified over a nickel column. The final material was stored in 500 mM NaCl, 125 mM imidazole, 50 mM TrisHCl, pH 7.9 at -20 °C until used. Protein concentration of granzyme A was determined by BCA assay (Pierce, Rockford, IL). The final yield of purified rGranA from 4L bacterial culture was 100 μg after enterokinase cleavage and purification. The purified rGranA yielded a single 52 kD protein, which formed a 28 kD monomer under reducing conditions. After enterokinase cleavage, the homodimer migrated with an apparent molecular weight 10 kD less than the proenzyme.

Example 2: Production of Inactive Mutated Human Granzyme A (S->ArGranA) in E. coli

This example illustrates generation of a recombinant inactive human granzyme A (S->ArGranA) by mutation of the active site Ser to Ala.

Mutation of the catalytic site Ser 184 to Ala was carried out via PCR mutagenesis. (Sambrook et al., Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989)). The mutated sequence was reamplified using a 5' primer containing the bacterial pel B leader sequence 5' of the coding sequence and the previously used 3' primer. The sequence was confirmed by dideoxy sequencing. The mutant protein (S->ArGranA) was expressed and purified as above without enterokinase cleavage. The mutant enzyme was designed so that the bacterial signal peptidase cleavage produced a protein whose N terminus coincided with that of the active enzyme. See Fig. 1. The mutant enzyme migrated with the same mobility as the enterokinase-cleaved rGranA. The final yield of the mutant protein was approximately 1 mg/4L.

Example 3: Production of Monoclonal Antibody and Polyclonal Antiserum against Human Granzyme A

This example illustrates the similarity of rGranA and S->ArGranA to native granzyme, by using monoclonal antibody CB9, produced towards native human granzyme, to immunoprecipitate rGranA.

Monoclonal antibody and polyclonal antiserum against human granzyme A were produced as follows. BALB/c mice were immunized with human granule proteins, extracted from human CTL clones by N_2 cavitation and Percoll density gradient centrifugation as described in Henkart et al., J. Exp. Med. 160:75-93 (1984). The primary immunization (60 μg protein in

complete Freund's adjuvant (CFA) in the footpads) was followed at 4 week intervals with i.p. injection of 75-100 μg granule protein in incomplete Freund's adjuvant (IFA). Three days after the second boost, splenocytes from an immunized mouse were fused to X63.653 myeloma cells for hybridoma production as described in Kohler and Milstein, Nature 256:495 (1975). Culture supernatants and ascites from hybridoma CB9 immunoprecipitated from human granules a 48 kD protein on nonreducing gels and a 28 kD protein on reducing gels, corresponding to the molecular weight of native human granzyme A. Polyclonal antisera was produced by immunizing mice with 10 μg rGranA in CFA and boosting 3 x with rGranA in IFA.

CB9 recognized a granzyme A conformational determinant since it did not react with native granzyme A in immunoblotting. Pro-rGranA, RgranA and S->ArGranA were specifically precipitated with CB9. Thus, the intact and mutated proteins adopted a common conformation which mimics that of native enzyme.

Example 4: Substrate Recognition and Enzyme Kinetics of rGranA

This example illustrates that the substrate specificity of rGranA is comparable to that of purified native human granzyme A. Michaelis Menton parameters were determined for known synthetic thioester substrates of native granzyme A.

The active site concentration of rGranA was determined by titration with p-nitrophenyl p-guanidino-benzoate (NPGB) by measuring the change in absorption at 412 nm for the initial acylation step production of p-nitrophenol (ϵ = 12,600 M^{-1} cm^{-1}). The number of catalytic sites was divided by two to give moles of granzyme.

After quantitation of active sites, rGranA was tested for cleavage of the synthetic thioester substrates Z-Arg-SBzl, Z-Lys-SBzl, Boc-Ala-Ala-Arg-SBzl and Boc-Trp-Arg-SBzl. Granzyme A activity was determined by measuring the hydrolysis of thioester substrates in 0.1 M Hepes, 0.01 M $CaCl_2$, pH 7.5 buffer containing 5% DMSO at 25°C in the presence of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) (Adachi et al., FEBS Letters 340:231-235 (1994)). Substrate stock solutions were prepared in DMSO and stored at -20°C. Initial rates were measured at 412 nm (ϵ_{412} = 14,150 M^{-1} cm^{-1}) using a Beckman DU-650 spectrophotometer when 10 μL of an enzyme stock solution was added to a cuvette containing 1.0 mL of buffer, 0.236 mM DTNB and 25 μL of substrate stock solution. After subtraction for background hydrolysis, duplicate measurements for each concentration were graphed on Lineweaver-Burk plots to determine the K_M, k_{cat} and k_{cat}/K_M for each substrate. Correlation coefficients for all plots were >0.99. Specific inhibitors (25 μl in DMSO) were added to 76 μM rGranA in 500 μl 0.1 M

Hepes, 0.01 $CaCl_2$ pH 7.5 at 25°C. Residual enzymatic activity was measured by assaying cleavage of 200 μM Z-Arg-sBzl substrate by 10 μl aliquots removed at various time points. First order inactivation rate constants (k_{obs}) were obtained by plotting in v_o/v_t versus time. Correlation coefficients were ≥0.98. Results are shown in Table 2.

Table 2

Kinetic Constants for the Hydrolysis of Thioester Substrates by Mouse Granzyme A and Human rGranA

Substrate	Kinetic Constant[a]	rGranA	Mouse Granzyme A[b]
Z-Arg-SBzl	k_{cat}	506	83
	K_M	177	315
	k_{cat}/K_M	2,860,000	260,000
Z-Lys-SBzl	k_{cat}	369	22
	K_M	767	130
	k_{cat}/K_M	481,000	170,000
Boc-Trp-Arg-SBzl	k_{cat}	66	
	K_M	170	
	k_{cat}/K_M	388,000	
Boc-Ala-Ala-Arg-SBzl	k_{cat}	83	45
	k_M	887	140
	k_{cat}/K_M	94,000	320,000
Boc-Ala-Ala-Asp-SBzl		NH[c]	NH
Boc-Ala-Ala-Met-SBzl		NH	NH

[a]The units of k_{cat}, K_M and k_{cat}/K_M are s^{-1}, mM and $M^{-1}s^{-1}$
[b]Data from Odake et al., Biochem 30:2217-2227 (1991).
[c]NH, no hydrolysis. The rGranA concentrations were 0.007-0.42 nM

The K_{cat}/K_M for cleavage of Z-Arg-SBzl by rGranA of 2.86×10^6 was more than 10-fold greater than that reported for purified granzyme A from human or mouse granules and was comparable to the activity of trypsin. For short substrates, Arg was preferred to Lys at the P1 site. The K_{cat}/K_M for the Arg substrate was approximately six fold higher than for the comparable Lys substrate. For the longer Arg substrates Boc-Ala-Ala-Arg-SBzl and Boc-Trp-Arg-SBzl, the K_{cat}/K_M values were 9.4×10^4 μM and 3.8×10^5 μM respectively. Typical

substrates for Aspase and Metase activity were not hydrolyzed. The S->A mutant and the proenzyme had no detectable tryptase activity against Z-Arg-SBzl.

Example 5: Inhibition Kinetics of rGranA

This example illustrates that the inhibition of rGranA by known inhibitors of granzyme A is comparable to that of purified native human granzyme A. RGranA activity was measured as described in Example 4.

3,4-dichloroisocoumarin (DCl), a general serine protease inhibitor (Harper et al., Biochem. 24:1831-1841 (1985), inhibited rGranA weakly with $k_{obs}/[I]$ of 15 $M^{-1}s^{-1}$. DCl also inhibited native human and mouse granzyme A weakly with $k_{obs}/[I]$ of 50 $M^{-1}s^{-1}$ (Odake et al., Biochem. 30:2217-2227 (1991)). The other three inhibitors (an isocoumarin (Ph-NHCONH-CiTEtOIC), a guanidinobenzoate (*p*-Gua$C_6H_4COOC_6H_4$-*p*CN), and a phosphonate ($C_6H_4CH_2SO_2$-Gly-Pro-(4-AmPhGly)$^P(OPh)_2$)) contain basic functional groups (isothioureido, guanidino, and amidino) and are more specific for trypsin-like enzymes. They inhibit rGranA much more potently with $k_{obs}/[I]$ values of 10^3-10^4 $M^{-1}s^{-1}$. Among these compounds, the isocoumarin derivative, Ph-NHCONH-CiTEtOIC, is the best inhibitor. All three compounds have been reported to be potent irreversible inhibitors for human recombinant granzyme A produced in S. cerevisiae. The inhibition constants of rGranA were comparable to those for native and yeast recombinant human granzyme A.

Example 6: RGranA Binds to the Cytoplasmic Proteins PHAP I and PHAP II

This example illustrates the isolation of two cytoplasmic proteins, PHAP I and PHAP II, that bind to rGranA. S->ArGranA was coupled to a matrix for affinity chromatography to isolate candidate substrates for granzyme A.

Purified S->ArGranA (4mg) was coupled to 1 ml of Affigel 10 (Biorad, Melville, NY) to produce an affinity column. Cytoplasmic extracts prepared from 1 x 10^9 K562 cells (ATCC) treated with NP40 lysis buffer (0.5% NP40, 25 mM KCl, 5 mM $MgCl_2$, 1 mM PMSF, 10 mM Tris HCl, pH 7.6) were applied to the column and eluted sequentially with 200 mM and 1 M NaCl in 50 mM TrisHCl, pH 7.6. Protein containing fractions were analyzed by SDS-PAGE. Fractions 13-15 and 21 were further purified by ion exchange chromatography on a Bioscale Q_2 column (Biorad, Melville, NY). The 200 mM NaCl eluate contained a 44 kD protein and the 1 M NaCl eluate contained a 33 kD protein, visible by SYPRO Orange staining. The 33 kD protein was probably present in the 200 mM fractions at lower levels since a band of this size

was visualized by silver staining. After ion exchange chromatography purification, the 33 and 44 kD proteins were subjected to tryptic digestion and peptide sequencing. The sequenced peptides showed nearly 100% homology to two previously coisolated proteins, PHAP I and PHAP II, isolated by binding to an HLA class II cytoplasmic peptide (Vaesen et al., Biol. Chem. Hoppe-Seyler 375:113 (1994)). The p33 tryptic fragment sequences, IPNLTHLNSGKN (SEQ. ID NO. 1) and DLSTIEPLK (SEQ. ID NO. 2), were identical to amino acids 87-98 and 102-110 of PHAP I, except that the initial I was a C in the PHAP I sequence. The p44 sequences, SGYRIDFYFDENPYFEN (SEQ. ID NO. 3) and EFHLNESGDPSSK (SEQ. ID NO. 4), were identical to amino acids 120-136 and 142-154 of cloned PHAP II.

To verify that the PHAP isolation from the column represented a physiologically significant interaction and to determine whether the PHAPs were substrates for granzyme A, the cell lysates were tested in a granzyme A cleavage assay. The cell lysates were treated for 1 hr at 30°C with 400 nM rGranA, S->ArGranA, pro-rGranA or buffer, and the reaction products were separated by SDS-PAGE on an 18% gel and transferred to nitrocellulose for immunoblotting. The immunoblot was probed with antiserum to an N terminal PHAP II peptide (Adachi et al., FEBS Letters 340:231 (1994)). The 44 kD PHAP II band was diminished and a new band of 25 kD was seen in lysates treated with active enzyme but not in samples treated with inactive variants. When the treated cell lysates were incubated with nickel resin to isolate His-tagged granzyme A and probed with PHAP II antiserum, PHAP II coprecipitated with the inactive mutant and proenzyme but not with the active rGranA. These results indicated that PHAP II was cleaved by active rGranA in cell lysates. PHAP II formed a stable complex with inactive variants of rGranA, but the active enzyme-substrate interaction was transitory and therefore was not visualized by coprecipitation.

Similar results were found when cell lysates treated with rGranA, S->ArGranA or buffer were immunoprecipitated with PHAP II antiserum, separated by SDS-PAGE and immunoblotted with polyclonal antigranzyme A antiserum. No rGranA coimmunoprecipitated with PHAP II in cell lysates as before, but a higher molecular weight complex of approximately 100 kD was observed when S->ArGranA was added to the cell lysates. This indicated that the PHAP II-mutant granzyme interaction was strong enough to withstand SDS denaturing conditions. Although higher molecular weight material immunoreactive with PHAP II antiserum was present in the different buffer conditions, the 100 kD complex was not seen. Its formation and stability may depend on ionic concentration and pH.

The cell lysate experiments were confirmed when pooled PHAP fractions 13-15 were

incubated for 4 hr with 100 nM rGranA or inactive pro-rGranA, PMSF-treated rGranA or S->ArGranA and analyzed by SDS-PAGE. The p33 PHAP I band after rGranA treatment was unchanged, suggesting it is not a substrate for granzyme A. After immunoblotting with PHAP II antisera against N terminal peptide (Adachi et al., FEBS Letters 340:231 (1994)), a cleavage product of 25 kD was visualized after treatment with active rGranA. After incubation with S->ArGranA, the PHAP II band disappeared and a new PHAP II-containing band at approximately 100 kD was again observed, confirming that PHAP II forms an SDS-stable complex with S->ArGranA.

Example 7: Importation of rGranA into Cell Nuclei and Resultant DNA Degradation

This example illustrates that rGranA is imported into cell nuclei and that this importation leads to DNA degradation.

RGranA or FITC-S->ArGranA was loaded into K562 or HL60 target cells by increasing membrane permeability with 0.3% Triton X-100. RGranA-treated cells underwent membrane blebbing and the blebs contained RNA (staining of blebs with propidium iodide was abrogated by treatment with RNase). Control experiments with thrombin or enterokinase loading did not show membrane blebbing. Similar target cell morphological changes have been described following CTL granule-mediated lysis and other forms of apoptosis (Sellins and Cohen, Immunol. Rev. 146:241 (1995)). Immunofluorescent staining with FITC-labeled S->ArGranA revealed diffuse localization of rGranA within the nuclei of loaded cells. This contrasts with the reported concentration of granzyme B within nucleoli of loaded cells (Trapani et al., J. Biol. Chem. 271:4127 (1996)). The importation of S->ArGranA, but not control protein, into isolated nuclei confirmed the nuclear localization by fluorescence microscopy.

Total cellular DNA harvested from rGranA treated cells revealed loss of high molecular weight DNA consistent with DNA degradation. However, there was no oligosomal laddering, as there was after treatment with anti-fas mAb (CH11). These results were confirmed by incubating isolated nuclei with rPHAP II in the presence or absence of rGranA and analyzing DNA by electrophoresis. High molecular weight DNA degradation was only seen after incubation with both PHAP II and granzyme A. Again no oligosomal laddering was seen, although a classic apoptotic ladder was found in DNA from nuclei incubated with rGranB produced by Baculovirus.

Example 8: Importation of PHAP II into Cell Nuclei and Cleavage of PHAP II to a 25 kD Fragment During Cytotoxic T Lymphocyte (CTL) Attack

This example illustrates that as a result of CTL attack, PHAP II migrates from the cytoplasm into the nucleus of a cell where it is cleaved.

To assess whether PHAP II is a physiological substrate for granzyme A, changes in PHAP II localization and cleavage during CTL attack were determined. CTL were allowed to form conjugates with their targets in the presence of EGTA, which inhibits Ca^{+2}-dependent granule exocytosis, and cells were stained for PHAP II by fluorescence microscopy before and after addition of Ca^{++}. PHAP II, normally located mostly in the cytoplasm, migrated to the nucleus of target cells within 15' of adding Ca^{++} to initiate cell death. When nuclear and cytoplasmic cell extracts obtained at timed intervals after Ca^{++} addition were analyzed for PHAP II by immunoblot with an antiserum to an N terminus of PHAP II, the translocation of PHAP II from the cytoplasm to the nucleus was detected beginning at 5 minutes. Within 20 minutes of the initiation of lysis, immunoreactive PHAP II was undetectable in the cytoplasmic fraction. A PHAP II-immunoreactive band, comparable in size to the 25 kD cleavage product of PHAP II by granA, was occasionally visualized only in the cytoplasm of control target cells, but within 5' of CTL attack a band of this size was visualized in the target cell nucleus. The 25 kD nuclear PHAP II immunoreactive band was only visualized if the potent selective granA inhibitor, Ph-HNCONH-CiTEtOIC, had been added prior to extraction, suggesting that the 25 kD cleavage product was rapidly further degraded, possibly by granA. The degradation of PHAP II during CTL attack was completely inhibited by Ph-HNCONH-CiTEtOIC.

Example 9: Cleaved PHAP II Complexes with a DNase

This example illustrates that cleavage of PHAP II by granA results in a 25 kD PHAP II cleavage product which forms a complex with DNase activity.

When purified rGranA is loaded into detergent-permeabilized cells or when target cells are targeted by IgE-directed rat basophil leukemia cells transfected with rGranA and perforin, target cell DNA degradation occurs (Hayes et al., J. Exp. Med. 170:933 (1989); Shiver et al., Cell 71:315 (1992)). These results were confirmed when rGranA was loaded by detergent permeabilization into K562 or HL60 cells. Loss of high molecular weight DNA, but not oligosomal laddering, occurs within 4 hours. When pet26b plasmid was incubated with rGranA alone there was no DNA degradation; however, the pooled PHAP fractions eluted from the S->ArGranA column caused degradation of the plasmid. To examine further whether the PHAP II

fraction has endonuclease activity, high molecular weight calf thymus DNA was incorporated into SDS-PAGE gels following the method of Rosenthal and Lacks, Biochem. 80:76 (1977), and the PHAP fraction proteins electrophoresed through the gel. After gradual renaturation and addition of Ca^{+2} and Mg^{+2}, incorporated DNA in the gel was visualized by ethidium bromide staining. Proteins with DNase activity degraded the incorporated DNA and did not stain. A single band of approximate apparent molecular weight 25 kD does not stain with ethidium bromide, which corresponds to the apparent molecular weight of the granA cleavage product of PHAP II. Since there was some spontaneous autolysis of purified PHAP II to an approximately 25 kD protein in solution, the 25 kD DNase in the PHAP II fraction might correspond to proteolyzed PHAP II.

To confirm that the cleaved PHAP II fraction contains a DNase, recombinant PHAP II was expressed without a periplasmic leader sequence and with a polyhistidine C terminal tag from the pet26b plasmid in E. coli, purified by nickel chelation chromatography and analyzed for DNase activity. The bacterial product contained two predominant PHAP II immunoreactive bands, full-length p45 and a 25 kD band, presumably the product of a bacterial protease. The rPHAP II cleaved plasmid DNA and its 25 kD degradation product degraded calf thymus DNA by SDS-DNA-PAGE analysis. When the SDS-DNA-PAGE gel was transferred to a membrane and immunoblotted with N terminal PHAP II antiserum, both full length p45 rPHAP II and a 25 kD band were visualized, but only the 25 kD band comigrated with the DNase activity. These results with rPHAP II confirmed that cleavage of PHAP II was required to activate DNase activity.

To confirm that granA activates PHAP II DNase activity, rPHAP II was expressed in the bacterial periplasm, which protects the recombinant protein from bacterial proteolysis. This product (rPHAP II_p) contained little or no detectable p25. When rPHAP II_p was incubated with rGranA, the full length protein was cleaved to major bands of 25 and 20 kD with minor products of 22, 12 and 8 kD, as analyzed by SDS-DNA-PAGE and anti-PHAP II (Western blot). Uncleaved rPHAP II_p had no detectable DNase activity, but the rGranA-cleaved rPHAP II_p complex contains a DNase. Control samples in which rGranA was pretreated with 250 mM Ph-NHCONH-CiTEtOIC, the potent and specific granzyme A inhibitor, before being added to rPHAP II_p showed no DNase activity on SDS-DNA-PAGE. This result verified that active serine esterase activity was essential for granzyme A activation of the PHAP II-associated DNase.

Although the DNase activity in the granzyme A-activated PHAP II complex has identical molecular weight to the granzyme A cleavage product of PHAP II (24 kD), the DNase activity

can be separated from immunoreactive PHAP II by ion exchange chromatography. Therefore, the PHAP II-associated DNase is a second 25 kD protein. Optionally, RNA is also present in the complex.

Example 10: Heat Shock Protein 27 Binds Tightly to rGranA

This example illustrates that heat shock protein 27 (hsp 27) binds tightly to rGranA, though it is not a substrate for cleavage by granzyme A.

To identify additional proteins that are involved in the granzyme A pathway, the K562 cytoplasmic lysate-loaded S->ArGranA column described in Example 6 was treated with progressively harsher eluting agents. No additional proteins eluted from the column with glycine-HCl or RIPA buffer, but 6M urea eluted two new proteins of 27 and 53 kD. These were analyzed by tryptic digestion and N terminal sequencing. The tryptic digests and MALDIS of the two proteins were identical, suggesting that p53 is a dimer of p27. Three p27 peptides (of 5, 8, and 10 amino acids) were identical to sequences of the heat shock protein hsp27. Merck et al., J. Biol. Chem. 268:1046-1052 (1993). The identification of p27 and p53 as the monomer and dimer of hsp27 were confirmed by immunoblotting with a polygonal anti-hsp27 rabbit antiserum.

The harsh elution conditions required to strip hsp27 from the granzyme column suggests a high affinity interaction. To confirm the interaction of hsp27 with granzyme A in cells and to determine whether hsp27 was a substrate of granzyme A, His-tagged active rGranA and inactive Pro-rGranA were added to K562 cell extracts and granzyme A was immunoprecipitated with nickel resin. When the immunoprecipitates were analyzed by SDS-PAGE and immunoblotted with hsp27 antiserum, the monomeric and dimeric hsp27 bands were visualized in samples to which rGran A or Pro-rGranA were added, but not in control samples to which no granzyme was added. These results verified the interaction of granzyme A and hsp27 and demonstrated that hsp27 was not a substrate for cleavage by granzyme A. Hsp27 may be involved in the morphological changes seen with granzyme A loading of target cells or in the transport of granzyme and/or the PHAPs to the nucleus during CTL attack.

Example 11: Production of Additional Monoclonal Antibodies and Polyclonal Antisera

The following antibodies and antisera have been produced according to the methods described in Kohler and Milstein, Nature 256:495-497 (1975): (i) polyclonal mouse and rabbit antisera to rGranA which react on Western blot to native and recombinant granzyme A (to complement the mAb CB9 generated to native granzyme A, which reacts in immunoprecipitation

but not in Western blot); (ii) polyclonal rabbit antisera to an N terminal peptide of PHAP II; (iii) polyclonal mouse antisera and at least eight monoclonal antibodies that react to rPHAP I by ELISA and on Western blot; (iv) two monoclonal antibodies to rPHAP II. In addition, mice have been immunized four times with rPHAP II (with sera ELISA titers >1:8000), so as to fuse spleens from immunized mice to produce PHAP II mAb. Commercial monoclonal antibodies to hsp27 are readily available from Stressgen, Victoria, BC.

Example 12: Immunoprecipitation, Coimmunoprecipitation and Immunoblot Methods

This example illustrates the immunoprecipition, coimmunoprecipitation and immunoblot methods used in certain of the other Examples described herein.

For immunoprecipitation, purified rGranA was added to 10 µl of CB9 ascites and 20 µl of a 70% slurry of Sepharose-Protein A (Pharmacia, Ascataway, NJ) in 300 µl of PBS and incubated for 30 min at 4°C. After washing, samples were boiled in SDS sample buffer and analyzed by SDS PAGE electrophoresis and stained with Coomassie blue.

For coimmunoprecipitation, 5 µg rGranA, S->ArGranA, or buffer was added to K562 cell lysate (1 x 10^6 cell equivalents) in 1 ml NP40 lysis buffer with 1% Triton X-100, previously precleared twice with preimmune rabbit serum and Protein A Sepharose. After incubation with 2.5 µl anti-PHAP II rabbit antisera and Protein A Sepharose for 2 hr at 4°C, the beads were washed extensively with RIPA buffer (150 mM NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris HCl, pH 7.6) and boiled in SDS-PAGE sample buffer for electrophoresis. The coprecipitation with nickel resin was performed similarly, except that cells were lysed in 0.1% NP40, 50 mM Tris HCl, pH 8, the preclearing was omitted, nickel resin was substituted for antiserum and Protein A Sepharose, and washes were in nickel binding buffer.

For immunoblotting, electrophoresed samples were transferred to nitrocellulose. After blocking with 5% non-fat dry milk, 0.05% Tween in TBS, the blot was incubated for 1 hr with 1/500 dilution of polygonal mouse anti-granzyme A or anti-PHAP II peptide polyclonal rabbit antisera (Adachi et al., FEBS Letters 340:231-235 (1994)), washed and incubated for 1 hr with 1/5000 donkey anti-rabbit HRP (Amersham, Arlington Heights, IL) and visualized by chemiluminescence with Luminol/Enhancer Solution (Pierce, Rockford, IL).

Those skilled in the art will be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for determining if an animal is at risk for a disease resulting in abnormal apoptosis, comprising:

providing an animal; and

evaluating an aspect of metabolism or structure of a serine protease or a serine protease binding molecule in said animal, an abnormality in said aspect of said metabolism or structure being diagnostic of being at risk for a disease resulting in abnormal apoptosis.

2. The method of claim 1 wherein said abnormal apoptosis results in activation of cell death.

3. The method of claim 1 wherein said abnormal apoptosis results in inhibition of cell death.

4. The method of claim 1 wherein said serine protease is a granzyme and said serine protease binding molecule is a granzyme binding molecule.

5. The method of claim 4 wherein said granzyme is granzyme A and said granzyme binding molecule is selected from the group consisting of PHAP I, PHAP II, a complex comprising PHAP II, and heat shock protein 27.

6. The method of claim 1 wherein said risk is a reduced risk as compared to a normal animal.

7. The method of claim 1 wherein said risk is an increased risk as compared to a normal animal.

8. A method for evaluating an agent for use in modulating apoptosis, comprising:

providing a cell;

providing an agent;

administering said agent to said cell in a therapeutically effective amount; and

evaluating the effect of said agent on an aspect of metabolism or structure of a serine protease or a serine protease binding molecule, a change in said aspect of said metabolism or structure being indicative of the usefulness of said agent in modulating apoptosis.

9. The method of claim 8 wherein said agent is administered to said cell in vitro, and if said change in said aspect of said metabolism or structure occurs, then further administering said agent to an animal in a therapeutically effective amount and evaluating the in vivo effect of said agent on an aspect of said metabolism or structure.

10. The method of claim 8 wherein said cell has a wild type pattern for said metabolism or structure.

11. The method of claim 8 wherein said cell or animal has a non-wild type pattern for said metabolism or structure.

12. The method of claim 8 wherein said serine protease is granzyme A and said serine protease binding molecule is selected from the group consisting of PHAP I, PHAP II, a complex comprising PHAP II, and heat shock protein 27.

13. The method of claim 8 wherein said agent comprises a serine protease, a serine protease binding molecule, or a biologically active fragment or analog thereof.

14. The method of claim 13 wherein said agent comprises granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, heat shock protein 27, or a biologically active fragment or analog thereof.

15. The method of claim 8 wherein said agent comprises a nucleic acid encoding a serine protease, a serine protease binding molecule, or a biologically active fragment or analog thereof.

16. The method of claim 8 wherein said agent comprises a nucleic acid encoding a regulatory sequence for a serine protease or a serine protease binding molecule, or a biologically active fragment or analog thereof.

17. The method of claim 8 wherein said agent is selected from the group consisting of a binding molecule for a serine protease or a serine protease binding molecule, and a binding molecule for a nucleic acid encoding a serine protease or a serine protease binding molecule.

18. The method of claim 8 wherein said agent is an antisense nucleic acid for granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, heat shock protein 27, or a biologically active fragment or analog thereof.

19. The method of claim 8 wherein said agent is a mimetic of granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, heat shock protein 27, or a biologically active fragment or analog thereof.

20. The method of claim 8 wherein said agent is a polyclonal or monoclonal antibody, or fragment thereof, that can immunoreact with granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27.

21. The method of claim 8 wherein said agent is selected from the group consisting of a natural antibody, a recombinant antibody, a chimeric antibody and a humanized antibody that can immunoreact with granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27.

22. The method of claim 8 wherein said agent is an antagonist or an agonist for granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, or heat shock protein 27.

23. The agent identified in claim 8.

24. A method for effecting apoptosis in a cell, comprising:

providing a cell which is deficient in effecting apoptosis;

providing an effective amount of an active endogenous DNase capable of degrading genomic DNA in said cell; and

administering said DNase to said cell so as to effect apoptosis.

25. The method of claim 24 wherein said endogenous DNase is a binding molecule for a serine protease.

26. The method of claim 24 wherein said endogenous DNase is a binding molecule for a serine protease binding molecule.

27. The method of claim 24 wherein said endogenous DNase is part of a complex comprising a binding molecule for a serine protease.

28. The method of claim 24 wherein said endogenous DNase is a complex comprising PHAP II.

29. The method of claim 24 wherein said effective amount of said active endogenous DNase is provided by providing PHAP II, a complex comprising PHAP II, or an active analog or fragment thereof, to said cell.

30. The method of claim 29 wherein said effective amount of said active endogenous DNase is provided by providing to said cell a nucleic acid encoding said PHAP II or an active analog or fragment thereof and expressing said PHAP II or said active analog or fragment thereof in vivo.

31. The method of claim 29 wherein said effective amount of said active endogenous DNase is provided by providing to said cell a nucleic acid encoding a component of said complex or an active analog or fragment thereof and expressing said component or said active analog or fragment thereof in vivo.

32. The method of claim 24 wherein said effective amount of said active endogenous DNase is provided by providing granzyme A or an active analog or fragment thereof to said cell so as to cleave PHAP II.

33. The method of claim 32 wherein said granzyme A is provided by providing to said cell a nucleic acid encoding said granzyme A or an active analog or fragment thereof and expressing said granzyme A or said active analog or fragment thereof in vivo.

34. The method of claim 24 wherein said effective amount of an active endogenous DNase is provided by providing heat shock protein 27 or an active analog or fragment thereof to said cell.

35. The method of claim 34 wherein said heat shock protein 27 is provided by providing to said cell a nucleic acid encoding said heat shock protein 27 or an active analog or fragment thereof and expressing said heat shock protein 27 or said active analog or fragment thereof in vivo.

36. A method for inhibiting apoptosis in a cell, comprising:

providing a cell which is capable of effecting apoptosis;

providing an agent which inhibits genomic DNA degradation in said cell by an endogenous DNase; and

administering said agent to said cell so as to inhibit apoptosis in said cell.

37. The method of claim 36 wherein said endogenous DNase is a binding molecule for a serine protease.

38. The method of claim 36 wherein said endogenous DNase is a binding molecule for a serine protease binding molecule.

39. The method of claim 36 wherein said endogenous DNase is part of a complex comprising a binding molecule for a serine protease.

40. The method of claim 36 wherein said endogenous DNase is a complex comprising PHAP II.

41. The method of claim 36 wherein said agent is monoclonal or polyclonal antibody.

42. A method for treating unwanted cell or infectious particle proliferation in an animal, comprising:

providing an animal having cells in need of treatment for unwanted cell or infectious particle proliferation;

providing an agent capable of activating apoptosis; and

administering said agent to said animal in a therapeutically effective amount such that treatment of said unwanted cell or infectious particle proliferation in said animal occurs.

43. The method of claim 42 wherein said unwanted cell proliferation results from a malignancy.

44. The method of claim 42 wherein said unwanted cell proliferation results from lymphoproliferation.

45. The method of claim 42 wherein said unwanted infectious particle proliferation results from a viral or bacterial infection.

46. The method of claim 42 wherein said apoptosis is mediated by granzyme A.

47. The method of claim 42 wherein said agent is a polypeptide.

48. The method of claim 47 wherein said polypeptide is selected from the group consisting of a serine protease, a binding molecule for a serine protease, and biologically active analogs and fragments thereof.

49. The method of claim 48 wherein said serine protease is a granzyme.

50. The method of claim 49 wherein said granzyme is granzyme A.

51. The method of claim 48 wherein said binding molecule for a serine protease is a granzyme binding molecule.

52. The method of claim 51 wherein said granzyme binding molecule is a granzyme A binding molecule.

53. The method of claim 52 wherein said granzyme A binding molecule is selected from the group consisting of PHAP I, PHAP II, a complex comprising PHAP II, and heat shock protein 27.

54. The method of claim 47 wherein said therapeutically effective amount of said polypeptide is administered by providing to said animal a nucleic acid encoding said polypeptide

or a biologically active fragment or analog thereof, and expressing said polypeptide in vivo.

55. The method of claim 54 wherein said nucleic acid is administered to said animal by introducing said nucleic acid into said cells of said animal ex vivo and administering said cells having said nucleic acid to said animal.

56. The method of claim 42 wherein said agent is a mimetic of a molecule selected from the group consisting of a serine protease, a binding molecule for a serine protease, and biologically active analogs and fragments thereof.

57. The method of claim 42 wherein said agent is an agonist of a serine protease or an agonist of a binding molecule for a serine protease.

58. A method for providing an animal having cancer with a therapeutic level of a polypeptide selected from the group consisting of granzyme A, a binding molecule for granzyme A, and biologically active analogs and fragments thereof, comprising administering to said animal a nucleic acid encoding said polypeptide.

59. The method of claim 58 wherein said binding molecule for granzyme A is selected from the group consisting of PHAP I, PHAP II, a complex comprising PHAP II, and heat shock protein 27.

60. A method for treating an animal at risk for unwanted cell or infectious particle proliferation, comprising:

providing an animal at risk for unwanted cell or infectious particle proliferation;

providing an agent capable of activating apoptosis; and

administering said agent to said animal in a therapeutically effective amount such that treatment of the unwanted cell or infectious particle proliferation occurs.

61. A pharmaceutical composition for treating unwanted cell or infectious particle proliferation in an animal, comprising:

a therapeutically effective amount of an agent capable of effecting apoptosis selected from the group consisting of a serine protease, a binding molecule for a serine protease, and

biologically active analogs and fragments thereof, and

a pharmaceutically acceptable carrier.

62. A pharmaceutical composition for treating unwanted cell or infectious particle proliferation in an animal, comprising:

a therapeutically effective amount of a recombinant nucleic acid encoding a polypeptide capable of effecting apoptosis selected from the group consisting of a serine protease, a binding molecule for a serine protease, and biologically active analogs and fragments thereof, and

a pharmaceutically acceptable carrier.

63. A method for treating an autoimmune disease or a transplant graft rejection in an animal, comprising:

providing an animal in need of treatment for an autoimmune disease or a transplant graft rejection;

providing an agent which inhibits apoptosis; and

administering said agent to said animal in a therapeutically effective amount such that treatment of said autoimmune disease or said transplant graft rejection occurs.

64. The method of claim 63 wherein said apoptosis is mediated by granzyme A.

65. The method of claim 63 wherein said agent is a polyclonal or monoclonal antibody, or a fragment thereof, that can immunoreact with a molecule selected from the group consisting of granzyme A, PHAP I, PHAP II, a complex comprising PHAP II, and heat shock protein 27.

66. The method of claim 63 wherein said agent is an antagonist of a serine protease or a binding molecule for a serine protease.

67. The method of claim 63 wherein said therapeutically effective amount of said agent is administered by providing to said animal a nucleic acid encoding said agent and expressing said agent in vivo.

68. A method for treating an autoimmune disease or a transplant graft rejection in an animal, comprising administering to an animal having an autoimmune disease or a transplant

graft rejection a therapeutically effective amount of an agent capable of inhibiting DNase activity of a complex comprising PHAP II or a component of said complex, or capable of inhibiting granzyme A cleavage of PHAP II.

69. A method for treating an animal at risk for an autoimmune disease or a transplant graft rejection, comprising:

providing an animal at risk for an autoimmune disease or a transplant graft rejection;

providing an agent capable of inhibiting apoptosis; and

administering said agent to said animal in a therapeutically effective amount such that treatment of the autoimmune disease or transplant graft rejection occurs.

70. A pharmaceutical composition for treating an autoimmune disease or a transplant graft rejection in an animal, comprising:

a therapeutically effective amount of an agent capable of inhibiting apoptosis by inhibiting a serine protease, a binding molecule of a serine protease, or biologically active analog or fragment thereof, and

a pharmaceutically acceptable carrier.

71. A pharmaceutical composition for treating an autoimmune disease or a transplant graft rejection in an animal, comprising:

a therapeutically effective amount of a recombinant nucleic acid encoding a polypeptide capable of inhibiting apoptosis by inhibiting a serine protease, a binding molecule of a serine protease, or a biologically active analog or fragment thereof, and

a pharmaceutically acceptable carrier.

72. A pharmaceutical composition for treating an autoimmune disease or a transplant graft rejection in an animal, comprising:

a therapeutically effective amount of cells wherein a recombinant nucleic acid encoding a polypeptide capable of inhibiting apoptosis by inhibiting a serine protease, a binding molecule of a serine protease or a biologically active analog or fragment thereof, has been introduced into the cells so as to express the polypeptide; and

a pharmaceutically acceptable carrier.

73. A method of degrading nucleic acid in vitro, comprising:

providing nucleic acid;

providing an isolated complex comprising PHAP II; and

contacting said nucleic acid and said complex in vitro such that said nucleic acid is degraded by said complex.

74. The method of claim 73 wherein said nucleic acid is provided in an infectious particle.

75. A method of degrading nucleic acid in vivo, comprising:

providing a cell having nucleic acid, said cell being deficient in active endogenous DNase;

providing a complex comprising PHAP II; and

contacting said nucleic acid and said complex in vivo such that said nucleic acid is degraded by said complex.

76. The method of claim 75 wherein said active endogenous DNase is for apoptosis.

77. A method for identifying an enzyme substrate, comprising:

providing an enzyme having an inactive active site;

providing a composition having a candidate substrate for said enzyme; and

contacting said composition with said enzyme and determining if said candidate substrate in said composition binds to said enzyme so as to identify said candidate substrate as a substrate for said enzyme.

78. The method of claim 77 wherein said enzyme is coupled to a matrix.

79. The method of claim 78 wherein said matrix is for affinity chromatography.

80. The method of claim 77 wherein said enzyme is a recombinant enzyme.

81. The method of claim 77 wherein said enzyme is a granzyme.

82. A bacterial recombinant expression vector comprising a nucleic acid encoding granzyme A, a leader sequence for periplasmic export of said granzyme A, and a carboxy terminal tag for purification of said granzyme A.

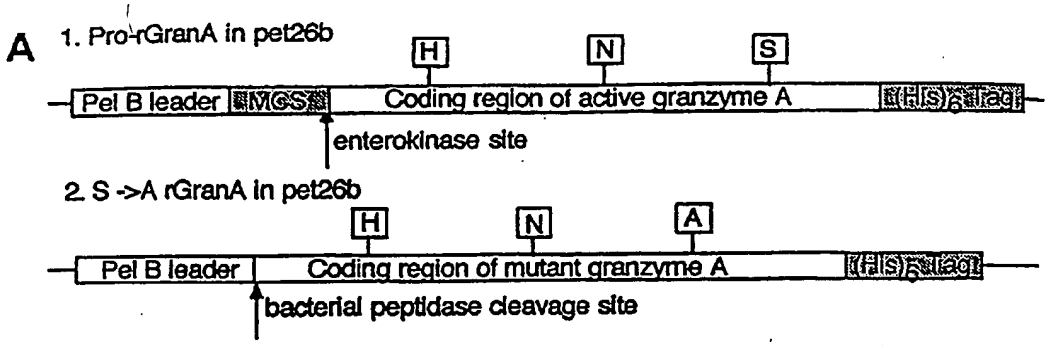
83. The vector of claim 82 further comprising an enterokinase site 5' of said nucleic acid encoding said granzyme A so as to enable in vitro activation of said granzyme A.

84. The vector of claim 82 wherein said granzyme A is human granzyme A.

85. A cell comprising said recombinant expression vector of claim 82.

86. A method for producing granzyme A comprising culturing a cell of claim 85 under conditions that permit expression of said granzyme A.

Fig. 1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Beresford, Paul J.

<120> METHODS FOR USING GRANZYMES AND BINDING MOLECULES THEREOF FOR TREATING DISEASES CHARACTERIZED BY ABNORMAL APOPTOSIS

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17022

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/02; C12N 1/38; A61K 38/10
US CL :435/23, 375; 514/13

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/23, 375; 514/13

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, CANCERLIT, SCISEARCH, BIOSIS, EMBASE, WPIDS
apoptosis, programmed cell death, serine protease, granzyme, phap-i, phap-ii, granzyme a, heat shock protein 27, hsp-27

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,017,489 A (PASTERNACK, et al) 21 May 1991, see the abstract and col. 2, in particular.	1-86
Y	US 5,578,705 A (SPRECHER) 26 November 1996, see the abstract and col. 1, in particular.	1-86
Y, P	BERESFORD, P.J., et al., Recombinant human granzyme A binds to two putative HLA-associated proteins and cleaves one of them. Proc. Natl. Acad. Sci. USA. August 1997, Vol. 94, pages 9285-9290, see entire document.	1-86
Y	SOWER, L.E., et al., Extracellular activaties of human granzyme A. J. Immunol. 1996, Vol. 156, pages 2585-2590, see entire document.	1-86

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 02 OCTOBER 1998	Date of mailing of the international search report 29 OCT 1998
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/17022

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WESTON, G.S., et al., Granzymes as potential targets for rational drug design. Curr. Med. Chem. 1996. Vol. 3, No. 1, pages 37-46, see entire document.	1-86
Y	SHI, L, et al., A natural killer cell granule protein that induces DNA fragmentation and apoptosis. J. Exp. Med. February 1992. Vol. 175, pages 553-566, see entire document.	1-86
Y	SMYTH, M.J., et al., Hypothesis: cytotoxic lymphocyte granule serine proteases activate target cell endonucleases to trigger apoptosis. Clin. Exp. Pharm. Phys. 1994. Vol. 21, pages 67-70, see entire document.	1-86.